

**STUDIES ON THE BIOACTIVITIES OF  
SELECTED EASTERN CAPE SEaweEDS**

**THESIS**

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## ABSTRACT

In studies on the bioactivities of selected eastern Cape seaweeds, a suitable extraction solvent, namely methanol was used. The antimicrobial, antineoplastic and antiviral activities were investigated, and a seasonal comparison of antimicrobial activities as well as a scanning electron microscopic study were included. A number of algae were found to display significantly high antibacterial activities, of these, the two red algae *Plocamium corallorhiza* and *Laurencia glomerata* and the two brown algae *Zonaria subarticulata* and *Ecklonia biruncinata* showed the most potent broad spectrum antibacterial activity.

*L.glomerata* and *E.biruncinata* were active against all five test bacteria, but were inactive against the yeast *Candida albicans*. *C.albicans* was the most resistant organism tested, with only *Peyssonelia capensis*, *P.corallorhiza* and *Ulva rigida* inhibiting its growth. Of the 17 seaweeds tested, none showed any clear seasonal changes in antimicrobial activity. The microorganisms however did vary in their susceptibility to treatment. *Staphylococcus aureus* and the *Micrococcus* species were the most susceptible to treatment by the algal extracts, with the Gram positive endospore former, *Bacillus subtilis* and the two Gram negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa* showing more resistance to treatment. *C.albicans* however was the most resistant organism. In the antineoplastic studies, the brown algae *Z.subarticulata*, *E.biruncinata* and *Sargassum heterophyllum* showed potent activity against both the normal, LLCMK, and cancerous, mouse melanoma-350 cells, reducing cell growth in each case. The green algae showed potent activity against the cancerous cells, lowering growth to 30% that of the normal cells. Normal cell growth was unaffected or was stimulated by the presence of these algal extracts. The red algae showed no antineoplastic activity. Representatives of each of the red, brown and green algae were used in the brine shrimp (*Artemia salina*) cytotoxicity study. None of the algae showed any toxicity towards the brine shrimp. In the antiviral studies against Polio Type 1, strain L-Sa, a reduction in virus infectivity was used as an indication of the presence of antiviral properties in the seaweeds tested. This was done by comparing the virus titres of algal-treated cells with those of untreated cells. Six of the algae tested displayed antiviral activity, these were the two Rhodophyceae *Hypnea spicifera* and *L.glomerata*, the two Phaeophyceae *Dictyopteris macrocarpa* and *Dictyota naevosa*, and the two Chlorophyceae *U.rigida* and *Halimeda cuneata*. Of these, *D.naevosa* showed particularly strong activity, recording a log reduction in virus titre of 4.

## CONTENTS

|                    |      |
|--------------------|------|
| List of Tables     | iv   |
| List of Figures    | v    |
| List of Appendices | vii  |
| Acknowledgements   | viii |

### CHAPTER 1

#### GENERAL INTRODUCTION, AIMS AND LITERATURE REVIEW

|        |                                                        |    |
|--------|--------------------------------------------------------|----|
| 1.1    | General Introduction                                   | 2  |
| 1.2    | Aims                                                   | 5  |
| 1.3    | Literature Review                                      | 6  |
| 1.3.1  | Antibacterial activity                                 | 6  |
| 1.3.2  | Antifungal activity                                    | 11 |
| 1.3.3  | Seasonality and distribution of metabolites            | 12 |
| 1.3.4  | Antineoplastic activity and toxicity studies           | 14 |
| 1.3.5  | Antiviral activity                                     | 16 |
| 1.3.6  | Secondary metabolites in terrestrial plants            | 19 |
| 1.3.7  | Secondary metabolites in microalgae and cyanobacteria  | 20 |
| 1.3.8  | Secondary metabolites in algae                         | 22 |
|        | (i) General                                            |    |
|        | (ii) Secondary metabolites active against fish         |    |
|        | (iii) Secondary metabolites active against echinoderms |    |
|        | (iv) Secondary metabolites active against molluscs     |    |
| 1.3.9  | Chemistry of active compounds                          | 25 |
| 1.3.10 | Ecological role of bioactive agents in algae           | 31 |

### CHAPTER II

#### THE EFFECT OF DIFFERENT EXTRACTION SOLVENTS ON SEAWEED BIOACTIVITY

|     |                        |    |
|-----|------------------------|----|
| 2.1 | Introduction           | 34 |
| 2.2 | Materials and Methods  | 35 |
| 2.3 | Results and Discussion | 38 |

### CHAPTER III

#### THE SEASONAL ANTIMICROBIAL ACTIVITIES OF SEAWEED EXTRACTS

|     |                        |    |
|-----|------------------------|----|
| 3.1 | Introduction           | 42 |
| 3.2 | Materials and Methods  | 43 |
| 3.3 | Results and Discussion | 44 |

## CHAPTER IV

### A CORRELATION OF SCANNING ELECTRON MICROSCOPIC INFORMATION AND ANTIBIOSIS

|       |                              |    |
|-------|------------------------------|----|
| 4.1   | Introduction                 | 58 |
| 4.2   | Materials and Methods        | 58 |
| 4.2.1 | Scanning electron microscopy |    |
| 4.2.2 | Critical point drying        |    |
| 4.2.3 | Metal coating                |    |
| 4.2.4 | Microorganism assay          |    |
| 4.2.5 | Gram stain                   |    |
| 4.3   | Results and Discussion       | 61 |

## CHAPTER V

### THE ANTINEOPLASTIC ACTIVITIES OF SELECTED SEAWEED EXTRACTS

|       |                            |    |
|-------|----------------------------|----|
| 5.1   | Introduction               | 67 |
| 5.2   | Materials and Methods      | 70 |
| 5.2.1 | Tissue Culture Procedure   |    |
|       | (i) Thawing of cells       |    |
|       | (ii) Splitting of cells    |    |
|       | (iii) Harvesting of cells  |    |
| 5.2.2 | Algal extracts             |    |
| 5.2.3 | <i>In vitro</i> Experiment |    |
| 5.2.4 | <i>In vivo</i> Experiment  |    |
| 5.3   | Results and Discussion     | 71 |

## CHAPTER VI

### BRINE SHRIMP ASSAY FOR ACTIVE CONSTITUENTS

|     |                              |    |
|-----|------------------------------|----|
| 6.1 | Introduction                 | 78 |
| 6.2 | Materials and Methods        | 79 |
|     | (a) Algae                    |    |
|     | (b) Sample Preparation       |    |
|     | (c) Hatching of Brine Shrimp |    |
|     | (d) Bioassay                 |    |
| 6.3 | Results and Discussion       | 80 |

## CHAPTER VII

### ANTIVIRAL ACTIVITIES OF SELECTED SEAWEED EXTRACTS

|     |                               |    |
|-----|-------------------------------|----|
| 7.1 | Introduction                  | 81 |
| 7.2 | Materials and Methods         | 83 |
|     | (a) Tissue Culture Procedure  |    |
|     | (b) Virus Propagation         |    |
|     | (c) Algal Extracts            |    |
|     | (d) Experimental Procedure    |    |
|     | (e) Virus Titre Determination |    |
| 7.3 | Results and Discussion        | 84 |

## CHAPTER VIII

### CONCLUSION

|              |      |
|--------------|------|
| Appendices   | ix   |
| Bibliography | xiii |

### List of Tables

|              |                                                                                               |
|--------------|-----------------------------------------------------------------------------------------------|
| Table 1.3.1  | A summary of some of the active compounds isolated from seaweeds.                             |
| Table 2.1.1  | The solvents used for the extraction of bioactive compounds.                                  |
| Table 2.2.1  | Algae collected from the First Sister, Riet River, on 5 March 1987.                           |
| Table 2.2.2  | Solvents used for antimicrobial testing.                                                      |
| Table 2.2.3  | Test organisms.                                                                               |
| Table 2.3.1  | The antimicrobial activity of selected seaweeds extracted in different solvents.              |
| Table 2.3.2  | A comparison of the effects of different solvents on seaweed bioactivity.                     |
| Table 3.1.1  | The seasonal patterns of antimicrobial production from seaweeds.                              |
| Table 3.1.2  | Contradictory results of antibiotic activity from various workers.                            |
| Table 3.2.1  | The seaweeds used in the seasonal antimicrobial activity study.                               |
| Table 3.3.1  | Antimicrobial activity of methanolic extracts from selected eastern Cape intertidal seaweeds. |
| Table 3.3.2  | A comparison of seasonal antimicrobial data.                                                  |
| Table 3.3.3  | A comparison of seasonal antimicrobial data for the three major algal groups.                 |
| Table 3.3.4  | The antimicrobial activity of the Rhodophycean species.                                       |
| Table 3.3.5  | The antimicrobial activity of the Phaeophycean species.                                       |
| Table 3.3.6  | The antimicrobial activity of the Chlorophycean species.                                      |
| Table 3.3.7  | A comparison of the most potent antimicrobially active seaweed extracts.                      |
| Table 3.3.8  | The bioactivity of the various orders of seaweeds tested.                                     |
| Table 3.3.9  | The susceptibility of test microorganisms to seaweed extracts.                                |
| Table 3.3.10 | A comparison of the susceptibilities of microorganism species to seaweed extracts.            |
| Table 5.1.1  | The anticancer activity of various algae                                                      |
| Table 6.1.1  | The uses of brine shrimp in general bioassays.                                                |
| Table 7.1.1  | Antiviral activity from compounds of marine origin.                                           |
| Table 7.3.1  | Algae yielding extracts toxic to Vero cells.                                                  |
| Table 7.3.2  | The virus titre reductions for the non-toxic algal extracts.                                  |

## List of Figures

- Fig. 1 The screening philosophy for marine natural product research [Baker, 1984].
- Fig. 2 The molecular structure of N-bromosuccinimide (NBS), and the possible active sites for halogenation in an algal enzyme.
- Fig. 3 The reaction of N-bromosuccinimide (NBS) and phenol to give 2,4,6-tribromophenol.
- Fig. 4 The proposed biogenetic relationship of avrainvilleol (ii) and 5'-hydroxyavrainvilleol (iii).
- Fig. 5 A graphic representation of the different solvent/seaweed bioactivity data (Table 2.3.2).
- Fig. 6 A graphic representation of the seasonal antimicrobial data (Table 3.3.2).
- Fig. 7 A graphic representation of the seasonal antimicrobial data (Table 3.3.3) for the three major algal groups.
- Fig. 8 A graphic representation of the antimicrobial activity (Table 3.3.4) of the Rhodophycean species.
- Fig. 9 A graphic representation of the antimicrobial activity (Table 3.3.5) of the Phaeophycean species.
- Fig. 10 A graphic representation of the antimicrobial activity (Table 3.3.6) of the Chlorophycean species.
- Fig. 11 A graphic representation of the susceptibility of the test microorganisms to seaweed extracts (Table 3.3.9).
- Fig. 12 A graphic representation of the susceptibility of the test microorganisms, as a group, to seaweed extracts (Table 3.3.9).
- Fig. 13 The micrograph showing the tip view of *Zonaria subarticulata*.
- Fig. 14 The micrograph showing the base view of *Zonaria subarticulata*.
- Fig. 15 The micrograph showing the tip view of *Ecklonia biruncinata*.
- Fig. 16 The micrograph showing the base view of *Ecklonia biruncinata*.
- Fig. 17 The micrograph showing the tip view of *Sargassum heterophyllum*.
- Fig. 18 The micrograph showing the base view of *Sargassum heterophyllum*.
- Fig. 19 The effect of the *Amphiroa anceps* extract on LLCMK (monkey kidney) and mouse melanoma-350 cells.
- Fig. 20 The effect of the *Spyridia cupressina* extract on LLCMK (monkey kidney) and mouse melanoma-350 cells.
- Fig. 21 The effect of the *Hypnea spicifera* extract on LLCMK (monkey kidney) and mouse melanoma-350 cells.
- Fig. 22 The effect of the *Zonaria subarticulata* extract on LLCMK (monkey kidney) and mouse melanoma-350 cells.

- Fig.23 The effect of the *Ecklonia biruncinata* extract on LLCMK (monkey kidney) and mouse melanoma-350 cells.
- Fig.24 The effect of the *Sargassum heterophyllum* extract on LLCMK (monkey kidney) and mouse melanoma-350 cells.
- Fig.25 The effect of the *Ulva rigida* extract on LLCMK (monkey kidney) and mouse melanoma-350 cells.
- Fig.26 The effect of the *Caulerpa filiformis* extract on LLCMK (monkey kidney) and mouse melanoma-350 cells.
- Fig.27 The effect of the *Codium duthieae* extract on LLCMK (monkey kidney) and mouse melanoma-350 cells.

## List of Appendices

### A Recipes used in Chapters II and III:

Phosphate Buffer (0,1M pH 7,5)  
Nutrient Broth  
Nutrient Agar  
Wickerham's Agar  
TTC plates (2,3,5-triphenyltetrazolium chloride)

### B Recipes used in Chapter IV:

Phosphate Buffer (0,1M pH 7,3)  
Buffered fixative (gluteraldehyde)  
Dehydrating agents  
Chloroform-based adhesive for SEM specimens

### C Recipes used in Chapter V:

Tissue culture medium  
Antibiotics  
Trypsin  
Procedure for freezing cells  
Tissue culture freezing medium  
Freezing cells

### D Recipes used in Chapter VII:

Fixative (0,01M PBS, pH 7,3 with Formalin)  
Stain (Crystal violet in ethanol)  
Titre Determination

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## CHAPTER 1

### GENERAL INTRODUCTION. AIMS AND LITERATURE REVIEW

#### 1.1 General Introduction

Seaweeds are highly adaptable and grow on practically every coastline throughout the world. They grow in tropical to near Arctic waters, and therefore have a wide temperature tolerance. They grow in distinct bands or zones in relation to the tidal levels, where they are subjected to wide fluctuations in humidity, temperature, salinity and light intensity. The pounding action of the waves and the abrasive action of the suspended sand particles which are churned up by the sea, are additional problems to which seaweeds must adapt. Some algae remain permanently submerged and so are not subjected to these extreme conditions [Branch and Branch, 1983].

The algae provide refuge for a wide variety of associated flora and fauna which feed on each other, and on the algae themselves. It has been found however, that many algae are able to control the amount of grazing by herbivores and the number of organisms and other plants that settle and grow on them, by the production of secondary metabolites. These secondary metabolites play a chemical defense role, the brown alga *Rafsia expansa* (J.Ag.) J.Agardh for example has a flat encrusting habit, and therefore exposes itself to attack by various herbivores. To overcome this problem, it produces antibiotic chemicals which kill settling organisms and deter herbivores [Branch and Branch, 1983].

Considerable interest has been shown in the effects that these secondary metabolites, produced by algae may have on human pathogens and other human ailments. Evidence for the use of marine algal products for the treatment of human ailments is extensive [Stein and Borden, 1984]. Agar, alginic acid and carrageenan are well known, and have long-standing and continuing applications. Oriental medicines have included algal extracts in their medications for centuries [Baker, 1984].

Some extensive studies have been carried out on the medicinal properties of seaweeds. The RRIMP (Roche Research Institute of Marine Pharmacology) screened 159 species, the success rate of which appeared to be encouragingly high. RRIMP found approximately 74% activity in the algae screened for *in vitro* antimicrobial activity conducted on crude extracts. This percentage was considerably reduced, to 2% for *in vivo* testing in microbiology, but was much higher in pharmacology. This study found the number of *in vivo* active extracts, fractions and pures to be encouragingly high [Baker, 1984].

Screenings of marine natural products have to be carried out methodically and with great care. The Roche Research Institute summarized their screening procedure for marine algae as follows. Fig.1 [Baker, 1984].



From the chart in Fig. 1, it is evident, that numerous steps have to be carried out before a natural product can seriously be considered worth pursuing. Experts in a number of fields are required, making natural product research both complex and extremely time consuming. A considerable amount of money, equipment, expertise and time is required for a single compound to be processed and tested to the stage of patient testing and later marketing [Baker, 1984].

To give some indication of the cost and time involved, an estimate in 1987 for the development of one new drug in the USA was \$100 million. On average 5 years is required for the completion of clinical trials in drug development, with tests of safety and efficacy accounting for 60% of this. Time allocations vary, however approximately 2 years are allocated for the completion of preclinical studies. After being reviewed and approved by the American Food and Drug Administration (FDA), drug application and clinical trials begin. This is a three phase program in which phase I involves the study of drug absorption and excretion; with drug levels in the blood being monitored, as are the drug concentrations in other body fluids. Small numbers of patients are required for this phase which takes between one and two years to complete. Phase II takes the same time, but involves comparisons of the efficacy of the new drug with those of an established drug used for the treatment of the same disease. Phase III has the same criteria as phase II, but requires large numbers of patients with the result that this phase takes 2-3 years. Successful clinical trials therefore require the enrolment of thousands of patients and the participation of large numbers of physicians [Gilbert, 1987].

Studies on medicinally active seaweeds have been carried out on the British, Californian, Oregon, Puerto Rican, Sicilian, Italian, Australian, Mediterranean, Caribbean and Pacific seaweeds, however no work has been done on seaweeds of the South African coastline. This work was therefore aimed at filling this gap.

## 1.2 Aims

The aims of this project were to:

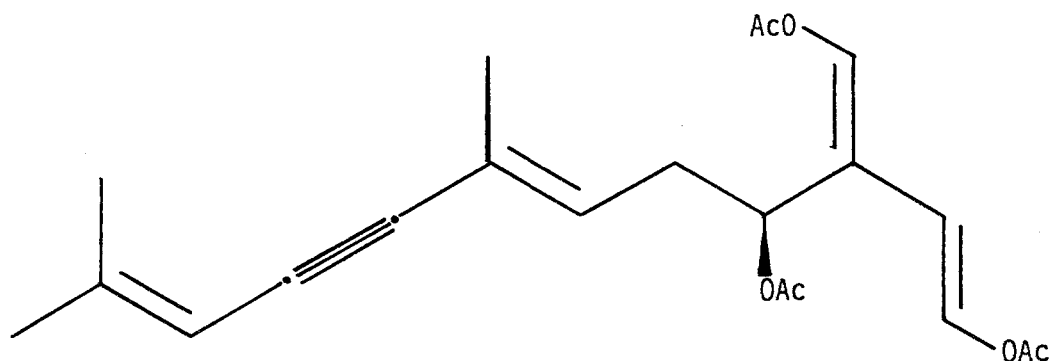
- (i) assess which solvents were most suitable for the extraction of biologically active secondary metabolites from seaweeds. This was necessary, as many different solvents have been used by other workers, resulting in varying results when the same seaweeds have been used. No explanation has been given by these workers as to why a particular solvent has been selected for their extraction processes.
- (ii) determine whether seaweed bioactivity changed seasonally. Seasonal changes in bioactivity have been shown to take place in some seaweeds. It was therefore necessary to determine whether this was or was not the case for the seaweeds studied.
- (iii) determine whether any seaweed extracts were particularly active against the microorganisms tested. As many organisms are becoming resistant to the substances used to eradicate them, any new compounds showing particularly high antimicrobial activities are of interest. Many of the active compounds used today were originally isolated from plants, animals, insects, seaweeds etc., it is therefore possible that a new source of compounds could be discovered from these same sources.
- (iv) assess the species assemblages of seaweeds i.e. class, order etc. to determine whether they showed bioactivity. Certain classes or orders of seaweeds have been shown to have high antimicrobial activity. It was of interest, to assess whether the same situation occurred in the seaweeds of the eastern Cape.
- (v) determine whether gram positive or gram negative bacteria were more susceptible to seaweed extracts. Many previous studies have shown the Gram positive bacteria to be more susceptible to treatment with seaweed extracts than the Gram negative bacteria. Occasionally however a strong anti-Gram negative bacterial activity is discovered, as these are particularly resilient organisms, these would be of interest.
- (vi) determine whether the antimicrobially active seaweeds were devoid of the test microorganisms in their natural habitat. As not much is known about the production of biologically active compounds by seaweeds, it was of interest to determine whether the surfaces of the seaweeds were free of those organisms against which they were active in the microbiological tests.
- (vii) determine the antineoplastic properties of a few selected seaweed species. Cancer research is being carried out extensively all over the world, hence there is always hope for the discovery of a new substance which would be of benefit. Three species from each of the three major algal groups were selected for testing against a cancerous cell line to assess their potential.
- (viii) determine the antiviral properties of a few seaweed species against Polio virus Type 1. As new outbreaks of Polio have occurred in the recent past, this virus is not fully under control. It was therefore of interest to determine the effects that selected eastern Cape seaweeds would have on this particular virus strain.

### 1.3 Literature Review

#### 1.3.1 Antibacterial Activity:

New drug development is extremely expensive, but is necessary, as many microorganisms become resistant to the drugs in use, largely due to their misuse. As mentioned in the introduction, it has been found that the percentage of marine algae exhibiting positive *in vitro* antimicrobial activity is very high; approximately 74%, making investigation worthwhile. The values for *in vivo* testing however are considerably lower, being approximately 2% in microbiology, with the pharmacological values being a lot higher than this [Baker, 1984].

Paul and Fenical (1987) in their continuing work on the order Caulerpales, have isolated a number of compounds. The major sesquiterpenoid metabolite to which activity has been attributed is caulerpenyne (1). This compound has been isolated from a number of species and was found to be responsible for antibiotic effects found in *Caulerpa prolifera* (Forsskal) Lamauroux specifically. Comparative tests were carried out using extracts of *C. prolifera* and purified caulerpenyne and the results were found to compare favourably [Hodgson, 1984, Paul and Fenical, 1987]. Of the organic extract obtained, 40-50% was caulerpenyne [McConnell *et al.*, 1982].



Compound 1 Caulerpenyne, a bioactive compound responsible for feeding inhibitions in fish, antibacterial activity and death in juvenile conch [Paul *et al.*, 1987].

The compounds isolated from the Caulerpalean algae appeared to have broad spectrum physiological activity, as they have been found to be effective against pathogenic microorganisms, they prevent settling of marine invertebrate larvae, and the same compounds reduce herbivory. The knowledge of this broad spectrum activity suggests that the metabolites have evolved against a number of detrimental factors and in so doing have increased the adaptive benefits of secondary metabolite production, and so enhance the survival ability of the algae in many ways. Of the natural products isolated from a broad spectrum of other algae, none have shown the potent activity reported from species of the Caulerpalean algae [Paul and Fenical, 1986].

Other members of the Chlorophyceae demonstrating promising activity towards human Gram positive pathogens include *Anadyomene menziesii* Harvey and *Anadyomene stellata* (Wulfen) C.Agardh. *A. stellata* was found, interestingly, to have an unknown compound present in the aqueous extracts, which showed selective antibacterial activity. The only phaeophyte from this Florida collection to show any antibacterial activity was *Spatoglossum schroederi* (C.Ag) Kutzing. This alga was found to be active against both the Gram positive organisms tested, i.e. *Staphylococcus aureus* and *Bacillus subtilis*.

Hornsey and Hide (1974) did a large study on the British seaweeds, and found many of these to possess antibacterial properties. One important factor that was established from this study, was that closely related species do not necessarily have similar activities. The two species which were studied, were *Chondus crispus* Stackhouse and *Gigartina stellata* (Stackh.) Batters; these two algae are from the same family. One was found to be highly active, and the other totally inactive. In this case, it was therefore not possible to make a correlation between taxonomic classification and antibiotic production [Hornsey and Hide, 1974].

Hornsey and Hide (1976 (a)) found that seasonal variations in antibiotic production did occur. The majority of the algae tested showed a winter peak of antibiotic activity, some showed summer and spring peaks, whereas others exhibited uniform activity throughout the year. Some discrepancies exist between the results obtained by Hornsey and Hide, and those obtained by other workers. This could well be due to specimens being collected at different times of the year [Hornsey and Hide, 1976]. These inconsistencies may also be due to the different reproductive and vegetative phases of the specimens exhibiting variations in antibiotic production.

These studies by Hornsey and Hide (1976 (a) and (b)) show that antimicrobial substances do exist in a large number of seaweeds and that they exhibit both temporal and spatial activity. Several precautions therefore have to be taken into account for both accuracy and uniformity to be maintained in screening studies.

According to Caccamese and Azzolina (1979), bioactivity is not uniformly distributed within the various orders. This became apparent in an extensive study carried out on the algae of the east coast of Sicily. Activity is concentrated in the Dictyotales and Fucales of the Phaeophyceae and the Ceramiales of the Rhodophyceae. The Cryptonemiales however, were nearly devoid of active species [Caccamese and Azzolina, 1979]. An extension of this study in southern Sicily confirmed these results, of the Dictyotales tested 75% of these showed activity, of the Fucales tested 60% showed activity and of the Ceramiales tested 44% showed biological activity. The Cryptonemiales and Gigartinales, both Rhodophyceae, accounted for small amounts of activity, 22% and 31% respectively [Caccamese *et al.*, 1985]. A similar distribution of activity was reported from the Mediterranean algae, even though the extraction methods differed [Pesando and Caram, 1984].

In these studies by Caccamese *et al.*, 1980 and 1985, the lipid extracts were tested against the Gram positive *B.subtilis* and the Gram negative *Escherichia coli*. The results showed strong activity, with *B.subtilis* being notably more sensitive to treatment than *E.coli* [Caccamese *et al.*, 1980]. Similar results were obtained for the French Mediterranean algae [Pesando and Caram, 1984]. The above two microorganisms appear to be the most sensitive and versatile organisms to work with.

Following the above study by Pesando and Caram (1984) a more detailed study was carried out on the Dictyotales (Phaeophyceae) specifically. Only the fertile specimens of *Dictyota dichotoma* f. *implexa* (Desfontaine) Gray were found to inhibit bacteria [Moreau *et al.*, 1984].

The conclusion that can be drawn from the above studies by Caccamese, Pesando, Moreau and their respective co-workers in separate studies, is that the distribution of antimicrobially active seaweeds is not uniform within the various orders. Of the Phaeophyceae the orders Dictyotales and Fucales show the

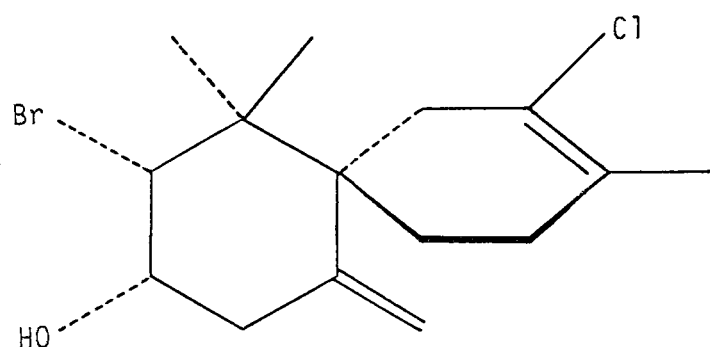
most activity, and within the Rhodophyceae, the order Ceramiales contains the most biologically active species.

To further broaden the survey the lipid-soluble extracts from the Caribbean algae were assessed by Ballantine *et al.* (1987). It was noted by Norris and Fenical (1985), that approximately 90% of algal secondary metabolites are lipid-soluble, hence their evaluation of lipid-soluble extracts only.

In this extensive study by Ballantine *et al.* (1987), in which 102 algae were tested, 64% showed antimicrobial activity. The results are not in agreement with those obtained by Burkholder *et al.* (1960) who found that only 44% of the algae tested showed biological activity. The results obtained by Burkholder *et al.*, however were obtained using aqueous extracts. The percentage activity therefore varied greatly. The algae showing significant inhibitory activity will be mentioned individually. The red alga *Asparagopsis taxiformis* (Delile) Trevisan showed particularly good activity against *B.subtilis*, *S.aureus*, *Pseudomonas aeruginosa* and *E.coli*. The aqueous extract of this alga, collected in Puerto Rico, also showed high antibiotic activity [Ballantine and Almodovar, 1977]. This activity has been attributed to the accumulated brominated compounds present in this alga [Fenical, 1974, 1975]. Another alga giving promising results was *Halimeda simulans* Howe. This alga was active against both Gram positive bacteria, and *E.coli*.

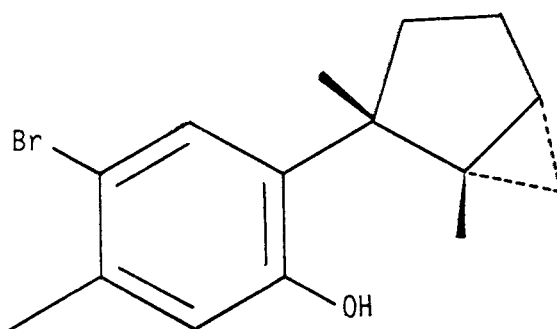
In this study by Ballantine *et al.* (1987), 61% of the extracts were found to be active against one or both Gram positive bacteria (*B.subtilis* and *S.aureus*). A much smaller percentage of the algae tested however, showed activity against the Gram negative bacteria (*E.coli* and *P.aeruginosa*), this being only 15%. Due to this low percentage, those showing strong activity are worth mentioning individually, these are, the green alga *Valonia ventricosa* J.Agardh, and the red algae *Gracilaria bursa-pastoris* (Gmel.) Silva, *Hypnea cervicornis* J.Agardh, and *Spyridia filamentosa* (Wulfen) Harvey. A number of other workers have found the majority of algae to show higher activity against Gram positive bacteria [Reichelt and Borowitzka, 1984, Allen and Dawson, 1960, Rao and Parekh, 1981].

Some of the algae examined by Ballantine *et al.* (1987) showed antimicrobial activity, whereas previous workers reported the absence of activity. This has happened in many cases, one example is that of *Laurencia obtusa* (Hudson) Lamouroux. The lipid extract of this species was shown to have limited activity against a single organism, whereas Norris and Fenical (1982) reported large amounts of the bioactive compound elatol (2). Variations in concentrations of the active halogenated sesquiterpenoids have been reported, as have variations in the antimicrobial activity. These variations have occurred in both Rhodophyceae and Phaeophyceae species [Ballantine *et al.*, 1987].

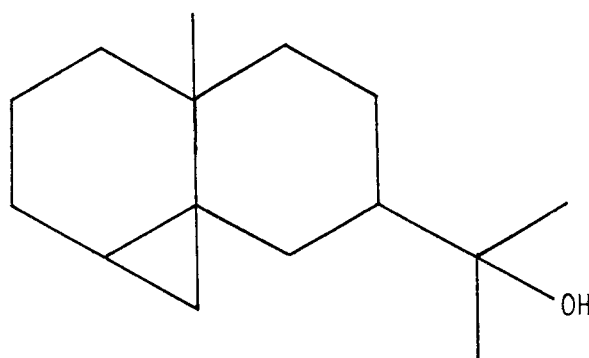


Compound 2 Elatol, exhibits antifungal activity, particularly against *Candida albicans*, and is toxic towards brine shrimp hatchlings [Brennan *et al.*, 1987].

Most of the reports so far are preliminary studies involving the testing of crude extracts. It is therefore necessary to isolate the substances responsible for the activity. Sims *et al.* (1975) isolated five active compounds from various algae which were tested against a number of organisms. The compound which compared favourably with the streptomycin sulfate control, was laurinterol (3). It completely inhibited the growth of the human pathogen *S.aureus*. The primary activity of this compound was against Gram positive bacteria, with only the compound cycloeudesmol (4) showing any inhibition against Gram negative bacteria [Sims *et al.*, 1975].



Compound 3 Laurinterol, active against Gram positive bacteria, activity compares with that of streptomycin sulfate [Sims *et al.*, 1975].

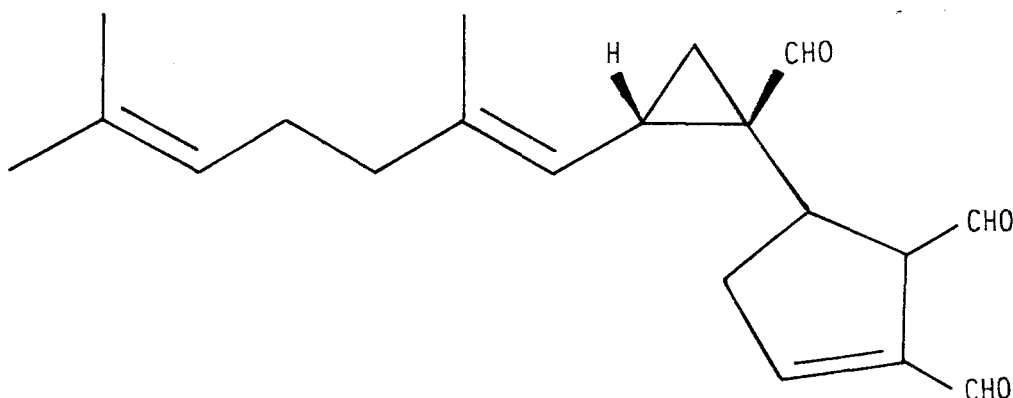


Compound 4 Cycloeudesmol, shows antimicrobial activity, and is active against Gram negative bacteria [Sims *et al.*, 1975].

Many halogen-containing metabolites show great promise in the biomedical field. Many of the red algae have been found to produce halogenated terpenoids and acetogenins (compounds produced by acetate polymerization) which show antimicrobial properties. The first red alga shown to have a high bromine content was *Rhodomela larix* C.Ag. which contained highly active brominated phenols [Fenical, 1975]. In contrast, the brown algae and particularly members of the families Dictyotaceae, Cystoseiraceae and Sargassaceae are known to produce complex diterpenoids and metabolites of mixed terpenoid- aromatic origins, but are not known to produce halogenated metabolites. Even so, these compounds have been found to display antimicrobial properties. Fenical and Paul (1984) concentrated on the metabolites produced by the highly bioactive terpenoids of the siphonaceous green algae of the family Udoteaceae. The genera *Udotea*, *Penicillus*, *Avrainvillea* and *Halimeda* fit into this family. Sesquiterpenoid and diterpenoid metabolites, most of which are linear or uncyclized, have been

extracted from these algae. They have the conjugated bis-enol functional group to which high biological activity is attributed. Little is known however about the interaction of these functional groups with biological molecules [Fenical and Paul, 1984].

The highly bioactive diterpenoid, halimedatrial (5) has been isolated from a number of Caribbean and Pacific species of *Halimeda*. This compound was found to be highly active against *S.aureus* and *B.subtilis*. The major isolate from the Caribbean alga *Udotea cyathiformis* Decaisne, also showed strong antimicrobial activity against the above two microorganisms, while the diterpenoid from *Penicillus dumetosus* (Lamour.) Blainville showed strong activity against *S.aureus* and *B.subtilis* [Fenical and Paul, 1984].



Compound 5 Halimedatrial, a highly bioactive diterpenoid, showing strong antibacterial, ichthyotoxic and cytotoxic activity [Fenical, 1984].

The occurrence of phenols within the brown algae results in many of the crude extracts having *in vitro* activity against Gram positive bacteria. These phenolic and polyphenolic compounds are tannins which are active defouling agents. They are toxic, as they bind and inactivate proteins [Baker, 1984].

Some of the brown algae tested by Baker (1984) showed significant antimicrobial activity. The lipid extracts of *Dictyota dichotoma* (Hudson) Lamouroux showed activity against *S.aureus*, *Streptococcus pyogenes* and *Streptococcus pneumoniae* as did the *Ecklonia radiata* (C.Agardh) J.Agardh and *Cystophora torulosa* extracts. These algae are members of the Dictyotales, Laminariales and Fucales orders respectively. The pharmacological analysis of the *D.dichotoma* extract showed it to be an oxidative-metabolism inhibitor [Baker, 1984].

Some research has also been carried out on the seaweeds of the Australian coast. These seaweeds are subjected to a great variety of conditions, as sea temperatures vary from tropical to near Antarctic [Baker, 1984]. Of the seaweeds studied from this region, the majority of lipid-soluble extracts of the red algal family Bonnemaisoniaceae, showed antimicrobial activity. The compounds showing particularly strong activity included three fibrolides isolated from *Delisea fimbriata* (Lamour.) J. Agardh. These were highly active against *S.aureus* and *E.coli*, with activity against *S.pyrogenes* and *S.pneumoniae* as well. These compounds however, were found to be toxic to mice [Baker, 1984].

The widespread occurrence of  $\omega$ 3 fatty acids in marine organisms is a unique feature of marine-derived lipids with considerable health and economic consequences. Relatively simple biochemical modifications of arachidonic and icosapentaenoic acids have been found to result in molecules possessing important hormonal and bioregulatory functions in mammalian systems. As a result of these observations, interest

has arisen and examinations have been made of metabolites of fatty acid origin from seaweeds collected off the Oregon coast, USA. This is part of an evaluation of the biomedicinal potential of marine plants. In this particular study, the lipid extract of *Ptilota filicina* J. Agardh was found to have strong activity against both Gram positive and Gram negative bacteria. Other species of the genus *Ptilota* from elsewhere in the world have also been examined and have shown great potential [Lopez and Gerwick, 1987].

One important consideration with regards to natural products in the biomedicinal and biocidal fields, is that rather than harvesting large quantities of marine flora for commercial extraction of the active compound, these natural products will serve as model systems for laboratory synthesis of new medicines and biocides. Because these new substances will be based on natural products, they should have the advantage of being biodegradable [Fenical, 1975].

### 1.3.2 Antifungal Activity:

As with the antibacterial work, Caccamese and Azzolina (1979) found the antifungal activities of the algae to be unevenly distributed within the various orders i.e. some orders had many active species, whereas others had few or none. Isolated incidences of bioactivity against the yeast *Saccharomyces cerevisiae* and the fungi imperfecti, *Penicillium digitatum* occur throughout the three major algal groups, but there is a concentration of activity within the Dictyotales and Fucales of the Phaeophyceae and the Ceramiales and Gigartinales of the Rhodophyceae. The organism *P. digitatum* appeared to be more sensitive to treatment than *S. cerevisiae* with particularly high activity being recorded against it by *Cystoseira elegans* of the Fucales and *Rytiphloea tinctoria* (Cem) C. Agardh. of the Ceramiales [Caccamese and Azzolina, 1979].

As a continuation of the above study, 24 lipid extracts of red and brown algae from the southern Italian coast were tested against the same yeast and fungal species. No inhibitions were recorded at all from algae of this area [Caccamese *et al.*, 1985].

Pesando and Caram (1984), in screening marine algae from the French Mediterranean coast, were in agreement with Caccamese *et al.* (1985), in that the Phaeophyceae, particularly the Dictyotales harbor more biologically active algae than do the Rhodophyceae, with the Chlorophyceae showing very limited activity. Of the algae tested, those worth noting include the activity of *Dictyota dichotoma* and *Dilophus fasciola* against the dermatophytes *Trichophyton mentagrophytes* and *Microsporum gypseum*, and the activity of *Dictyota linearis* C. Agardh against the moulds *Sporothrix schenkii* and *Aspergillus fumigatus*, as well as *T. mentagrophytes* [Pesando and Caram, 1984]. Of the Rhodophyceae examined, only three species proved to be active. These were *Hypnea musciformis* (Wulfen) Lamouroux, *Falkenbergia rufolanosa* (Harvey) Schmitz and *Laurencia obtusa* (Hudson) Lamouroux, all of which showed specific activity against *T. mentagrophytes* [Pesando and Caram, 1984]. A non-isoprenoid cyclic ether, laurencyenine was isolated from *L. obtusa*. This was active against *T. mentagrophytes*, and therefore could be the active compound present [Caccamese and Azzolina, 1980].

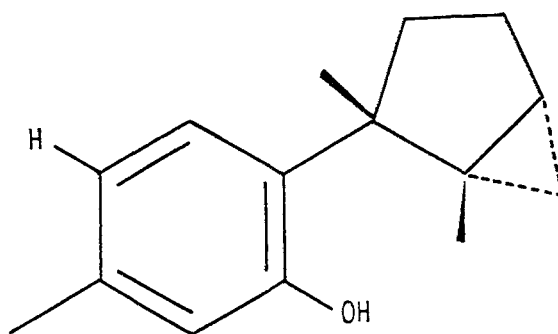
Following the above study, several genera of the Dictyotales (Phaeophyceae) from the same area were selected for examination. The species were collected at different times of the year to compare the activity of fertile versus non-fertile plants. It became apparent from this, that the fertile algae showed more potent activity.

All the Dictyotales tested exhibited broad spectrum antifungal activity, however the activity of these did seem to vary seasonally, the *D.dichotoma* spring extract was active against all the dermatophytes tested, the yeast *Candida albicans* and the mould *Aspergillus fumigatus*, whereas the summer extract inhibited the dermatophytes only. Analysis of the active fractions suggested that the active components could be terpenoid in nature [Moreau *et al.*, 1984].

Hodgson (1984), looking at the biological potential of the south Florida seaweeds, found only one member of the Chlorophyceae which showed any activity against the human pathogen, *C.albicans*. This alga, *Caulerpa verticillata* J.Agardh showed no activity against any of the other organisms tested [Hodgson, 1984].

An extensive study was carried out by Ballantine *et al.* (1987) in which the lipid extracts of 102 species from the Puerto Rican waters were evaluated. The activity against the yeast *C.albicans* was limited, with the Phaeophyceae giving the highest percentage activity. In general, 19% of the extracts tested showed activity, with only two of these being worth mentioning, the red alga *Asparagopsis taxiformis* which gave exceptionally good inhibition and the brown alga *Dictyopteris justii* Lamour [Ballantine *et al.*, 1987].

Sims *et al.* (1975) did more extensive studies to isolate the active substances. They examined the secondary metabolites from a number of algae. *Laurencia pacifica* Kylin yielded laurinterol (3) and debromolaurinterol (6) both of which showed noteworthy activity against two strains of *C.albicans*, as did cycloeudesmol (4) from *Chondria oppositoclada* Dawson. These results were comparable with the inhibition levels of the antibiotic streptomycin sulfate. It should be noted, that the similar inhibitory activity shown by laurinterol and debromolaurinterol indicates the lack of activity contributed by the bromine atom [Sims *et al.*, 1975].



Compound 6 Debromolaurinterol, showed activity against *Candida albicans* [Sims *et al.*, 1975].

The genus *Laurencia* has been found generally to produce a number of novel compounds which confer a special ecological advantage on the alga. The compound elatol (2) isolated from *L.obtusa* was also found to exhibit modest antifungal activity towards *Cladosporium cucumerinum* [Brennan *et al.*, 1987].

### 1.3.3 Seasonality and Distribution of Metabolites:

Seasonal variations in antibiotic production occurs in some marine algae. Hornsey and Hide (1976 (a)) carried out an extensive study in which it became apparent, that four major patterns of production exist. These are, firstly, the *Polysiphonia* type, in which antibiotic production occurs uniformly throughout the year e.g. *Polysiphonia lanosa* (Linnaeus) Tandy. This uniform production could indicate that the

antibiotics are merely secondary metabolites from some essential, major metabolic pathway. The second type is the *Laminaria* type, in which a winter peak of activity occurs. This period coincides with the period of minimum photosynthesis and general metabolic inactivity of the plant. Algae which fit into this group include, *Chondrus crispus*, *Laurencia pinnatifida* (Turner) Lamouroux, *Ulva lactuca* Linnaeus, *Laminaria digitata* (Linnaeus) Edmonson and *Laminaria saccharina* (Linnaeus) Lamouroux. Thirdly, the *Dictyota* type; this is characterised by a summer peak of antibiotic activity. This period coincides with a period of warmer water temperatures and increased light intensity. The intensity of herbivory probably also increases, as does the number of spores, larvae etc. Members of this group include *Dictyota dichotoma* and the red alga, *Dilsea carnosa*. The fourth group is the *Codium* type showing a spring peak of activity. This activity is associated with increased metabolic activity and includes *Codium fragile* (Suhr) Harvey and *Halidrys siliquosa* Lyngbye. These studies by Hornsey and Hide (1976 (a)) were carried out in the northern hemisphere.

Eleven of the species found previously to possess antibacterial properties were selected for further investigations. Of these, four were shown to have periods of total inactivity during the year. These included *U.lactuca* (March-August), *L.saccharina* (May-October), *D.dichotoma* (December- February) and *Ascophyllum nodosum* Stackhouse (September-May). It should be noted, that these are northern hemisphere seasons. These results are not fully in agreement with previous works. The results of *U.lactuca* do not agree with those of Pratt *et al.* (1951), who, working on *Ulva linza* Linnaeus, found that minimum activity occurred during winter. Similarly, Vacca and Walsh (1954) reported a spring maximum and a winter minimum in activity for *A.nodosum*. Almodovar (1964) found that *L.obtusa* exhibited maximum activity from November to April, with no activity at all being detected during certain periods of the year. In contrast, the studies by Hornsey and Hide (1976 (a)) indicated antibiotic production throughout the year for *L.pinnatifida*.

The overall variation in production of antimicrobial substances shown by the above study by Hornsey and Hide (1976 (a)) makes it difficult to correlate this phenomenon with biochemical and/or physiological processes. It is therefore necessary for the active principle(s) to be identified first, before this can be done. These seasonal variations partly account for some of the apparently contradictory results obtained by previous workers using samples collected at one period of the year only [Hornsey and Hide, 1976 (a)].

Another observation which could also partly account for the discrepancies between the results of Hornsey and Hide (1976 (a) and (b)) and other workers, is the variation in antibiotic production of algae in different phases of reproduction. Different algal groups exhibit different reproductive trends of antibiotic production. The Rhodophyceae exhibit four trends which include:

- (i) enhanced activity within the tetrasporic phase, in comparison with the other phases
- (ii) enhancement in the cystocarpic phase
- (iii) similarly high activities in the cystocarpic and sterile phases
- (iv) similarly high activities in the tetrasporic and sterile phases.

The Phaeophyceae exhibit different patterns of activity in which three trends have emerged, these being:

- (i) similar levels of activity in both the sporophyte and gametophyte generations
- (ii) similar levels in both the sterile and gametophyte plants
- (iii) high antibiotic activity within the sterile thallus, and no activity in the reproductive sporophyte.

Further studies by Hornsey and Hide (1976 (b)) on the distribution of antibiotic activities within the algal thallus also gave a wide variety of results. Six algal species, already known to produce antibiotics were examined. From these studies, some trends emerged and it appears, that most algae fit into one of three groups. Firstly, uniform distribution throughout the algal thallus e.g. *U.lactuca*; secondly, maximum activity in the meristematic or younger tissues, e.g. *C.crispus*, *D.carnosa* and *C.fragile*; and thirdly, maximum activity in the older parts of the thallus e.g. *L.saccharina*. Even though these three general groups have emerged, some algae have a far more complex distribution of activity. *L.digitata* for example, the activity decreases slightly in the lamina just behind the meristem, but then increases towards the older parts of the blade. Experiments done at quarterly intervals showed no significant seasonal variation in antibiotic distribution within the above six species. Due to the variable results obtained, no correlation could be drawn between antibiotic activity and meristematic and/or non-meristematic regions. These different patterns of distribution make it possible to believe that numerous compounds may be responsible for the observed antimicrobial activities in seaweeds.

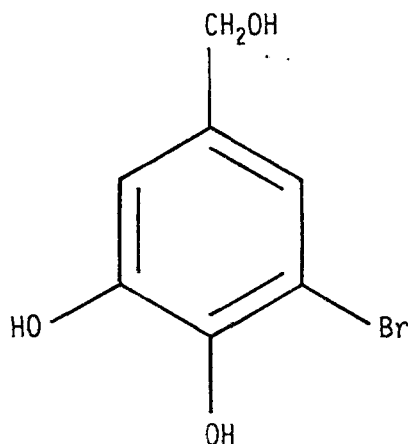
The seasonal variations in activity that occur, the varying distribution of activity within an individual thallus, the variability depending on the reproductive phase of the thallus, and the general distribution of antimicrobial activity within the different orders makes it necessary for several precautions to be taken for accurate screening of algae for antimicrobial activities.

#### 1.3.4 Antineoplastic Activity and Toxicity Studies:

Until recent years, the only knowledge of seaweeds being used for the treatment of human cancer was that of Chinese herbal medicines. As the Chinese have been using seaweed decoctions for human cancer treatment, it seemed obvious to start research with those that had already been tried. The two members of the Phaeophyceae which were used extensively were *Sargassum* and *Laminaria* species [Yamamoto *et al.*, 1974]. A preliminary study using these genera involved the isolation and testing of compounds obtained against two cell lines, namely mouse sarcoma-180 and leukemia tumours. Inhibition of these tumours did occur and further studies showed that it appeared to be a host-medicated reaction by a nondialyzable polysaccharide. Another study confirmed these *in vitro* results [Yamamoto *et al.*, 1981]. Gerwick *et al.*, (1987) in an attempt to isolate the active compound, isolated two compounds from *L.obtusa*, both of which were devoid of any cytotoxic activity [Gerwick *et al.*, 1987]. Even though the active compound was not isolated, the initial work showed great promise, and stimulated further research into the potential of marine natural products as antineoplastic agents [Stein and Borden, 1984].

Hodgson (1984) carried out a study on the Florida seaweeds using two tissue culture cell lines, human epidermal carcinoma and mouse lymphocytic leukemia. Three of the Chlorophyceae species, *Anadyomene menziesii*, *A.stellata* and *Caulerpa prolifera*, extracted in chloroform gave strong positive results. As most of the biological activity found in green algae has been traced to lipids such as terpene derivatives, activity would be expected in the chloroform extract. What was interesting however, was the high activity detected in the aqueous extracts of both *A.menziesii* and *A.stellata*. The compound responsible for this activity however, was not identified. *C.prolifera* showed general toxicity which may have been due to the sesquiterpenoid, caulerpenyne (1) [McConnell *et al.*, 1982; Hodgson, 1984]. The activity demonstrated by caulerpenyne is considered strong by the standards of the National Cancer Institute [Hodgson, 1984].

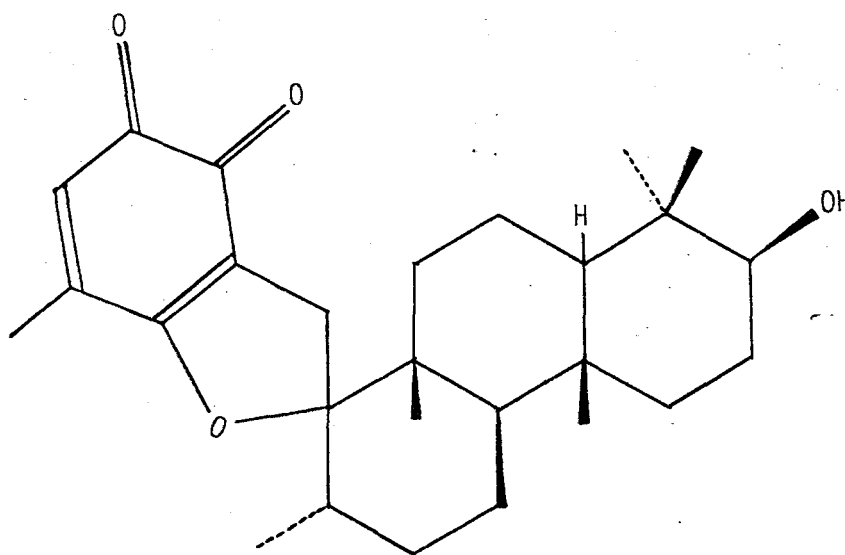
Another green alga showing significant cytotoxicity is *Avrainvillea nigricans* collected from the Puerto Rican waters. The compound isolated, 3-bromo-4,5-dihydroxybenzyl alcohol (7) showed noteworthy activity against the human epidermal carcinoma cell line [Colon *et al.*, 1987]. These results were more promising than those reported by Hodgson (1984).



Compound 7 3-bromo-4,5-dihydroxybenzyl alcohol, showed activity against human epidermal carcinoma cells [Colon *et al.*, 1987].

In further studies, the marine algae from a number of the Pacific islands were shown to be active against either lymphocytic leukemia or Ehrlich ascites tumour cells, or both, in mice [Kashiwagi *et al.*, 1980]. From all the results obtained, it was not possible to make correlations between activity and taxonomic grouping, and no studies using the antitumour compounds on human subjects have been carried out [Stein and Borden, 1984].

Although the Chlorophyceae appear to have gained most attention, the other groups have also been studied and have shown potential. Of the Phaeophyceae, crude extracts of members of the Dictyotaceae have been shown to be highly bioactive and have shown promising antitumour and cytotoxic activities [Kelecom and Teixeira, 1986]. One example is the brown alga, *Stypopodium zonale* (Lamouroux) Papenfuss which was found to contain a compound called stypoldione (8). This compound when tested was found to be relatively non-toxic to mice, and expanded the life expectancy of mice injected with P388 lymphocytic leukemia cells or Ehrlich ascite tumour cells. This result is significant, but requires further investigation, as stypoldione may inhibit the growth of dividing cells, which is an undesirable effect for continuous treatment [O'Brien *et al.*, 1984].



Compound 8 Stypoldione, an ortho-quinone mono alcohol which increases the life expectancy of mice injected with tumours [O'Brien *et al.*, 1984].

It appears that in general, seaweeds are far richer in antineoplastic agents than terrestrial plants, even though this is a newer field of study. Marine natural products made their first appearance in clinical trials at the National Cancer Institute (USA) relatively recently. The first compounds were the didemnins and bryostatins. In the near future, many more compounds will be at the stage of clinical trials, as this is a promising and expanding new field [Cardellina II, 1986].

From these studies, it is apparent, that algae have great potential in the field of cancer research. A considerable amount of research is still necessary, as many algae are toxic to normal cells. Compounds are required which are effective against cancer cells, but allow the continued, normal functioning of the normal cells. Any blockages of DNA replication, cell division, RNA synthesis, enzyme functioning, etc. would be undesirable, as the cell would malfunction as a result. Selectively active compounds are therefore essential for the effective treatment of cancer.

#### 1.3.5 Antiviral Activity

A number of antiviral compounds have been isolated from marine algae, most of which have been found to be polysaccharide in nature. Gerber *et al.*, (1958) noted that when insoluble compounds were suspended in agar and injected into embryonated eggs infected with Influenza B virus, protection was minimal and erratic. The results obtained also varied depending on the batch of agar used. It was therefore necessary to test the agar-producing, *Gelidium cartilagineum* (Linnaeus) Gaillon, and the carrageenin-producing, *Chondrus crispus*. The aqueous extracts of these two seaweeds were tested as the possible producers of the antiviral activity found. The extracts which gave positive test reactions typical of polysaccharides were composed of D-galactose units principally. These extracts were active against Influenza B and mumps viruses in embryo eggs, but strangely were inactive against Influenza A and Newcastle disease virus.

In further studies, Kathan (1965) using extracts of kelp, isolated a glycoprotein which inhibited neuraminidase and so prevented the attachment of the influenza virus. The extract was protective to embryonated eggs as late as 6 hours postinfection and reduced the virus infectivity titre. Purification of the extract did not increase the activity and as a result, the exact nature of the inhibition was not determined.

Some extensive studies followed these initial works. Deig *et al.* (1974) studied two related species collected from the Californian coast, *Cryptosiphonia woodii* (J.Ag.) J.Agardh and *Farlowia mollis* (Harvey and Bailey) Farlow and Setchell. Extracts of these seaweeds were tested against Herpes Simplex Virus-1 (HSV-1), HSV-2 and eleven other viruses. These extracts were found to be active against herpes virus, however there was no direct inactivation of the virus, nor was interferon induced; neither was any activity found against the other viruses tested.

Ehresmann *et al.* (1977) continued this work and tested 28 species against the same broad spectrum of mammalian RNA and DNA viruses, as well as three strains of HSV. Six members of the Rhodophyceae were found to contain antiviral substances which caused greater than a two log reduction in the infectivity of HSV-1 and -2. In addition, anti-Coxsackie B<sub>5</sub> virus activity was detected in extracts of *Constantinea simplex* Setchell. Since many of the active algae were members of the family Dumontiaceae (Rhodophyta: Cryptonemiales), in an additional examination, members of this family were studied only. Four out of the five species studied showed anti-HSV activity.

It is significant that most of the active algae were members of the single family Dumontiaceae. These algae which are morphologically related and similar anatomically, contain antiviral substances which appear chemically similar. Unpublished data of Ehresmann *et al.* (1977) has shown the response of active anti-HSV extracts of *Nienburgia andersoniana* (Ag.) Kylin and *Cryptopleura violacea* (Ag.) Kylin, (Ceramiales) to various physical and chemical treatments. These differ from those of the Dumontiaceae, suggesting the presence of at least two separate classes of antiviral substances.

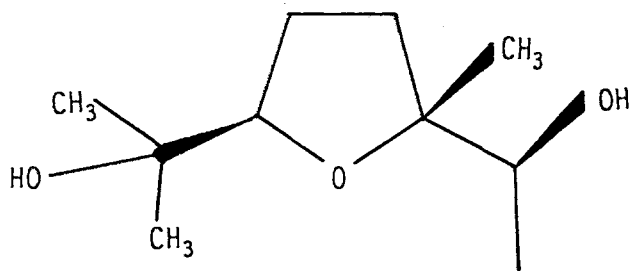
The physical and chemical properties of the anti-HSV extracts of *F.mollis* and *C.simplex* indicated that a polysaccharide was responsible for the activity (Ehresmann *et al.*, 1977). It is interesting to note at this stage, that the active agents of *G.cartilagineum* (Turn.) Grev. and *C.crispus*, previously reported, were also polysaccharides [Gerber, 1958]. The inhibition of encephalomyocarditis (EMC), ECHO, Coxsackie Ag and HSV have also been reported by both natural and synthetic acid polysaccharides [Takemoto and Fabisch, 1964].

The following study by Richards *et al.* (1978) is a continuation of the work carried out by Ehresmann *et al.* (1977). *C.simplex* and *F.mollis* were used, and were tested against mouse embryo fibroblast cells and also on experimental infections in mice. Treatment of the cells before viral inoculation resulted in effective inhibition of the replication of HSV-1, HSV-2, vaccinia virus and vesicular stomatitis virus (VSV), but not EMCV, Semliki Forest virus (SFV), or murine cytomegalovirus (MCMV). This viral inhibition was found to be greater with pretreatment than with posttreatment of cells. In fact when therapy was initiated after, or at a site other than that of viral inoculation, no significant effect on mortality or mean death day was observed. Neither algal preparation was effective on mice inoculated intraperitoneally with EMCV, SFV or MCMV, nor in animals infected intravaginally with HSV-2.

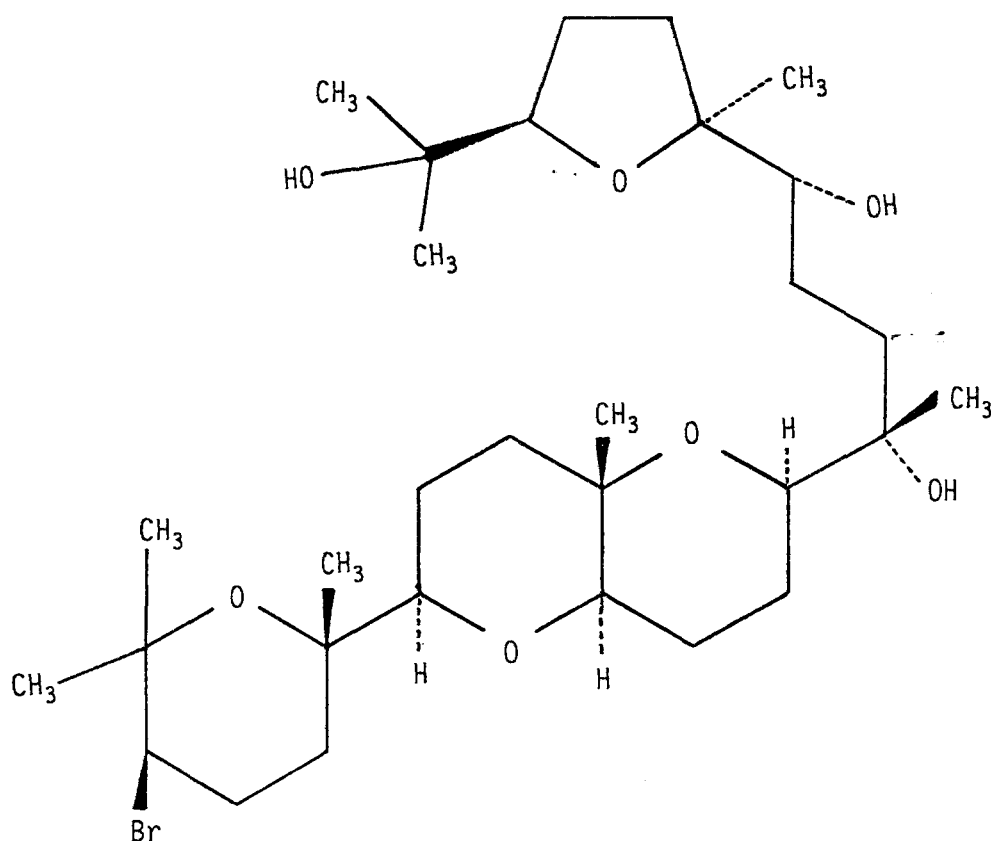
The active antiviral substance again appears to be a polysaccharide. It has been postulated that these substances exert their effect by blocking or coating receptor sites necessary for viral attachment to the cells. This is supported by the evidence that the antiviral effect is lost if treated monolayers are washed before viral inoculation. The observation that treatment of the monolayers after viral inoculation was in general less effective than treatment before viral challenge, also supports this postulation.

The prophylactic but not therapeutic antiviral efficacy of these compounds seriously limits their potential use in treatment of human HSV infections. The fact that the crude extracts when given parenterally or locally after the establishment of infection lack effect, suggests that they would be ineffective in the treatment of recurrent HSV infections, generalized infections or HSV encephalitis in humans [Richards *et al.*, 1978].

Sakemi *et al.*, (1986) in a later study of algae in the Okinawan waters discovered that a crude extract of the red alga *Laurencia venusta* displayed significant activity against VSV and HSV-1. The active components were identified as thysiferol (9), Thysiferyl-23 acetate and venustatriol (10). Venustatriol was an unknown compound, but was found to be a new tetracyclic ether derived from squalene [Sakemi *et al.*, 1986].



Compound 9 Thysiferol, isolated from *Laurencia venusta*, showed antiviral activity [Sakemi *et al.*, 1986].



Compound 10 Venustatriol, a tetracyclic ether derived from squalene, which shows anti-VSV and anti-  
HSV-I activity [Sakemi *et al.*, 1986].

Algae from the eastern coast of Sicily were tested by Caccamese *et al.* (1980) for antiviral activity against Tobacco Mosaic Virus (TMV). A large number of the algae tested were found to possess slight anti-TMV activity with two in particular showing significant activity. These two were both members of the Phaeophyceae, *Dictyota dichotoma* var. *intricata* (C.Ag.) Grev. and *Taonia atomaria* (Woodw.) J.Ag. Identification procedures yielded lipid extracts devoid of polysaccharides or proteins, both of which are often responsible for activity against mammalian viruses. This suggests, that the active agents against the plant virus TMV may be unusual lipophyllic metabolites [Caccamese *et al.*, 1980].

Work has been carried out on antiviral compounds, especially those active against the herpes simplex virus. In the treatment of HSV and most other viral infections, it is necessary to obtain a compound which is effective used locally or given parenterally after the establishment of the virus. So far, this has not been successful with the HSV. Although more work is required in the antiviral field of algal medicines, breakthroughs have been made which show great potential.

#### 1.3.6. Secondary Metabolites in Terrestrial Plants:

It is known, that terrestrial plants contain over 12 000 different natural products of varied biogenetic origins, including alkaloids, terpenoids, acetogenins and aromatic compounds. It is known, that those compounds possessing aldehyde groups are among the most toxic and deterrent compounds known. Warburganal, polyodial and isovelleral, all terpenoid aldehydes, and the iridoid aldehydes, are potent toxins which are produced by both terrestrial plants and insects [Paul and Fenical, 1986].

Even so, a number of natural products isolated from terrestrial plants display significant biomedical properties. The formosan plant, *Psychotria rubra*, which is known in Chinese folk medicines, showed significant, reproducible inhibitory activity against human epidermal carcinoma cells. A new naphthoquinone named psychorubrin and the known halenalin were isolated as the active principles [Hayashi *et al.*, 1987].

Another plant used extensively in China since ancient times, is that of a *Tripterygium* species which is a perennial twining vine. The roots of this plant have been used in folk medicines for the treatment of cancer and inflammatory diseases, and were shown to exhibit significant antitumour activity against the human epidermal carcinoma cell line [Hori *et al.*, 1987].

Our knowledge of biomedicinally useful natural products was broadened by a study carried out by Zemek *et al.* (1987) on 25 low molecular weight compounds of natural origin. Eleven of these, all of which were lignin biosynthetic precursors and products of thermal, alkaline and acid degradation of lignin during wood processing, when tested against various organisms, gave significant results. Isoeugenol was the most potent effector in all cases, it possesses an  $\alpha,\beta$ -side chain double bond and a  $\beta$ -positioned methyl group. This was found to be a potent antimicrobial compound [Zemek *et al.*, 1987].

Another group of compounds which have gained particular interest, are the natural pyrrolizidine alkaloids (P.A.s). These have potentially hazardous effects on mammals, including man, and plants containing P.A.s represent hepatotoxic, mutagenic and carcinogenic materials to grazing animals. On the otherhand, some of these compounds were found to possess tumour- inhibitory action, and indicine N-oxide, a P.A. of low toxicity has been used in clinical trials. From these studies, it appears that only plants which contain unsaturated P.A.s can produce significant degrees of cytotoxicity [Wassel *et al.*, 1987 (c)].

Terrestrial plants have been studied quite extensively for medicinal properties, particularly the oils obtained from many of the common herbs and spices. It was observed, that in the presence of clove and cinnamon, for example, neither the growth of *Aspergillus parasiticus*, nor the production of aflatoxins was observed, whereas the essential oils of garlic, onion, oregano, savoury and thyme were effective against several yeasts causing food spoilage [Jansen *et al.*, 1987]. From a pharmacological point of view, the essential oils of fennel, chamomile, peppermint and thyme have all been used successfully in clinical trials for external applications [Jansen *et al.*, 1987].

This report makes it clear, that great potential does exist within the terrestrial plants, as a great diversity of plant types and active compounds exist.

### 1.3.7 Secondary Metabolites in Microalgae and Cyanobacteria:

The microalgae are a biochemically diverse group of microorganisms which include the cyanobacteria and nearly a dozen eukaryotic classes. The microalgae are known to produce a wide array of biologically active compounds, amongst these, are antibiotics. The algal antibiotics are largely unidentified, but are known to include fatty acids, other organic acids, bromophenols, other phenolic inhibitors, tannins, terpenoids, polysaccharides, other carbohydrates and alcohols [Metting and Pyne, 1986].

One of the earliest references to algal use in medicine is that cited by Brunswig in 1500 AD, of *Nostoc* (Cyanobacterium) used in the treatment of gout and cancer [Metting and Pyne, 1986].

It has become clear from a number of studies, that microalgae release many kinds of substances both actively and passively while living, upon death and during subsequent decomposition. In a study by Fabregas and Veiga (1984) on the effects of exo- and endocellular products from two marine microalgae, the secreted products of *Navicula laevissima* in the stationary phase, were tested against human pathogenic organisms, and were found to be active against *E.coli*, *Sarcina lutea* and *B.subtilis*. It appears therefore, that a microalgal population can control the organisms present in its environment to a certain extent. The antibacterial properties of sea water, reported by Moebus (1972) may therefore be due, at least partially, to the antibacterial compounds produced by marine microalgae in the water [Moebus, 1972, Fabregas and Veija, 1984, Fabregas *et al.*, 1986].

Since the original work by Pratt (1942) on the antibacterial activity of chlorellin, a substantial amount of work has been done on the antibiotic production of phytoplankton. Lustigman (1988), studied four strains of the marine alga *Dunaliella*, two from high pollutant waters, and two from low pollutant waters. The results of these studies showed that a broad spectrum antibiotic was produced by the algae growing in the high pollution areas, with no such production in the low pollutant zones. These algae therefore flourish in environments which contain high concentrations of bacteria and other organisms e.g. coastal water rich in organic nutrients and biomass [Lustigman, 1988].

The cyanobacteria have recently received considerable attention by academic researchers, the National Cancer Institute and industry. Gerwick *et al.*, (1986) recently undertook a study of this group as a potential source of novel bioactive natural products. In a survey, the novel styrylchrome structure, hormothamnione, isolated from *Hormothamnion enteromorphoides* Grunow was found to be a potent cytotoxin to several human cancerous cell lines, and appeared to operate via inhibition of RNA synthesis [Gerwick *et al.*, 1986].

Recently, the compound tubercidin which was isolated and identified from *Tolypothrix byssoidea*, was shown to display *in vitro* activity against human epidermal carcinoma and NIH/313 cells, and *in vivo* activity against P-388 lymphocytic leukemia in mice. Another compound, L-asparaginase from *Chlamydomonas* species, inhibited the growth of lymphosarcoma inoculated into CH3 mice [Metting and Pyne, 1986].

During investigations by Kobayashi *et al.* (1988) on bioactive substances from marine organisms, extracts from the laboratory cultured dinoflagellate *Amphidinium* species, were examined. The first 25-membered macrocyclic lactone, amphidinolide C which was isolated, showed potent antineoplastic activity against L 1210 murine leukemia cells *in vitro*. In previous studies, two antineoplastic macrolides, amphidinolide A<sup>12</sup> and -B<sup>13</sup> were also isolated from the same dinoflagellate [Kobayashi *et al.* 1988]. Amphidinolide-A exhibited antitumour activity against both L 1210 and L 5178Y murine leukemia cells *in vitro* [Kobayashi *et al.*, 1986].

As a source of pharmaceutical products, the microalgae have gained less attention than seaweeds, due to the difficulties associated with dealing with this type of material. It is also known, that the presence and concentration of bioactive materials vary with environmental fluctuations and with the age of the organism. However because microalgae can be cultured in controlled environments (fermenters) for the production of high-value specific chemicals, it is possible to optimize conditions for production of a given

product. The ability to control conditions suggests that these organisms may become the focus of screening and selection for biologically active compounds [Metting and Pyne, 1986].

### 1.3.8 Secondary Metabolites in Algae:

#### (i) General

Algae inhabit zones of intense stress with respect to dessication, irradiation, wave action, grazing etc. and as a result, it is necessary for them to adapt to ensure survival. This is done in many cases by the production of chemical deterrents or secondary metabolites with antibiotic properties. This reduces or prevents grazing, the settling of microorganisms, algal spores, sessile animals, crustacean larvae etc. on the algal surfaces. All of the above would reduce light availability to the host plant and so reduce photosynthesis, interfere with water motion and hence reduce nutrient and gaseous exchange, increase drag on the plant and as a result reduce growth and reproductivity. The production of antibiotic chemicals is carried out by many algae e.g. *Ralfsia expansa* [Branch and Branch, 1983].

In chemical variation studies, comparisons were carried out on the amounts of defense chemicals found in different parts of the plant. It was expected, that the holdfasts would contain less of the active compound, as they are less susceptible to herbivory. This however does not occur in all cases, as specific allocation of the defense compounds would be genetically expensive to the alga due to its coenocytic construction. One example where variation does occur is that of *Avrainvillea nigricans*. The blade of this alga was found to have greater quantities of secondary metabolites than the stipe and haptera [Colon *et al.*, 1987].

The extent of herbivory varies in different habitats e.g. tropical reef areas have higher levels of herbivory than do the adjacent seagrass beds. This made it possible to study chemical production variations under varying degrees of stress from herbivores. In this study, the reef populations were found to have higher concentrations and greater varieties of metabolites than the grassbeds. Further studies would be required however, before it would be possible to determine whether secondary metabolite production is induced under increased levels of herbivory, or if other environmental factors are responsible for variations [Paul and Fenical, 1986].

It appears also, that temporal and/or geographic variations in secondary metabolite production occur. *A.nigricans* collected from the Puerto Rican waters yielded defense compounds, whereas the species from the Caribbean were devoid of halogenated organic metabolites and bromoperoxidase activity. Similarly, *Avrainvillea longicaulus* (Kutzing) Murray and Boodle collected from the Caribbean and western Pacific gave variations in metabolite biosynthesis [Colon *et al.*, 1987].

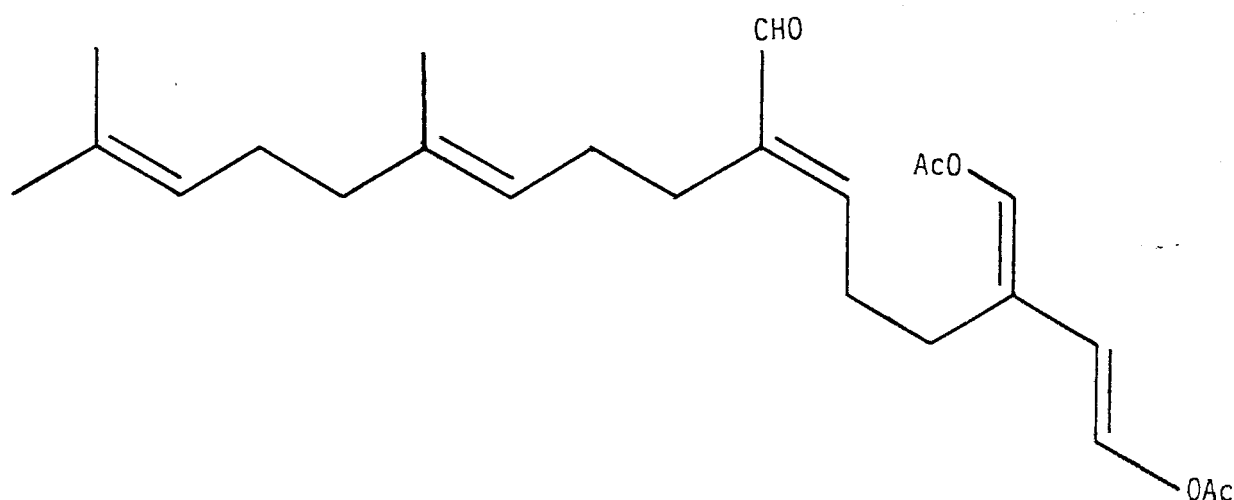
Amino acids have been isolated from seaweeds e.g. the amino acid taurine from *Ptilota pectinata* (Gunner) Kjellman collected from Norway. The British species *Ptilota plumosa* Agardh yielded potent hemagglutinin activity with human B cells, and the species *P.filicina* from the USSR has very high concentrations of essential amino acids which have been examined as a potential food source [Lopez and Gerwick, 1987].

(ii) Secondary Metabolites active against fish:

The genus *Caulerpa* has received special attention due to its abundance world-wide, and its apparent immunity to herbivory. Paul and Fenical (1982) isolated three compounds from *Caulerpa bickinensis* W.R. Taylor, two of which showed biological activity when tested against the Pacific damselfish, *Pomacentrus philippinus* (Evermann and Seale) [Paul and Fenical, 1982].

Paul *et al.* (1987) in a continuation of their previous work on the genus *Caulerpa*, carried out a field feeding preference study on 12 species of *Caulerpa*. The species *Caulerpa ashmeadii* Harvey was found to be the least preferred by herbivorous fishes, and in some cases was not eaten at all. The activity of the isolated compounds was found to be far higher than that of caulerpenyne (1) and caulerpicin [Paul *et al.*, 1987], however, caulerpenyne has been identified as a bioactive compound responsible for some feeding inhibitions [McConnell *et al.*, 1982].

An experiment carried out by Lobel and Ogden (1981) using the parrotfish *Sparisoma radicans* showed the low survival of these fish when fed *Caulerpa mexicana* Sonder ex Kuetzing, *Halimeda incrassata* (Ellis) Lamouroux or *Penicillus pyriformis* (A. and E.S. Gepp). This was attributed to the toxins present in the *Caulerpa* species, and the calcium content of the latter two. Considering the fact that the parrotfish is adapted for eating calcareous matter, the mortality of the fish cannot be attributed to the high calcium content, but to the presence of toxic terpenoids in all three species. These results compare favourably with mortalities observed in juvenile conch which were fed diets including the toxic terpenoids caulerpenyne (1), halimedatrial (5) and udoteal (11) [Lobel and Ogden, 1981].



Compound 11 Udoteal, a terpenoid showing ichthyotoxic activity, and also causing 50% mortality of juvenile conch within 6 days [Paul and Fenical, 1986].

Previous studies by Sun and Fenical (1979) on a member of the family Udoteaceae, *Rhipocephalus phoenix* (Ellis & Solander) Kuetzing, revealed the presence of two related sesquiterpenoids. One of these, rhipocephalin was found to display significant avoidance behaviour in herbivorous fish [Sun and Fenical 1979].

Ichthyotoxicity tests were carried out on a number of brown algae as well. The compounds that have been isolated in these studies include the compound stypoldione (8) which was isolated from the alga *Stypopodium zonale*. This alga was found to be toxic to fish in aquaria and it was this compound that

was found to be responsible for the effect [O'Brien *et al.*, 1984]. The species *Dilophus guineensis* (Kützinger) J. Agardh of the order Dictyotales collected in the Caribbean was found to contain two types of diterpenes. The xenicane-type diterpenoid, dilophic acid was found to cause the loss of righting reflex in the common goldfish (*Carassius auratus*) ichthyotoxicity test [Schlenk and Gerwick, 1987].

Another example of ichthyotoxicity in algae reveals a strange relationship between the alga *Dictyota crenulata* J. Agardh and the sea hare *Aplysia vaccaria*. The typical metabolites, of which acetoxycrenulide is of interest, have been isolated from both of the above sources, and is found in the digestive glands of the sea hare. This compound possesses ichthyotoxic properties, and so plays a fundamental role in the defense strategy of the mollusc, and also assists the alga. This is evident in the fact that *D. crenulata* has colonized the infralittoral zone world-wide, yet has no mechanical defenses against predation [Kelecom and Teixeira, 1986].

(iii) Secondary Metabolites active against Echinoderms:

Paul and Fenical (1982) carried out a considerable amount of work on the effect that members of the order Caulerpales had on sea urchin egg fertility. Their initial work was carried out on the Pacific alga *Caulerpa bikiensis* from which they isolated two monocyclic sesquiterpenoids, diacetate and dialdehyde, both of which were highly effective at very low concentrations [Paul and Fenical, 1982].

In a continuation of this work, some new diterpenoids were isolated from members of the Caulerpaceae and Udoteaceae families. These algae are closely related and have been shown to produce metabolites which have a unique functional group which is believed to be responsible for their bioactivity. Of the compounds isolated exoxylacetone 1, a linear diterpenoid possessing an enol acetate functionality and a linear diterpenoid were found to completely inhibit cell division of fertilized sea urchin eggs at low concentrations [Paul and Fenical, 1985].

In 1986, Paul and Fenical carried out further studies on the order Caulerpales using a wide range of toxicity assays, these included the sea urchin (*Lytechinus pictus* Verrill) fertilized egg cytotoxicity test, the sea urchin (*L. pictus*) sperm assay and the sea urchin (*L. pictus* and *Echinometra mathaei* de Blainville) larval toxicity assay. The reasons for the development of these toxicity assays, was because the Caulerpalean algae have been shown to be of low preference in the diets of herbivores in previous feeding preference studies [Littler *et al.*, 1983]. No full definition for the proliferation and abundance of these algae has ever been given, but it is generally accepted, that the calcification provides a physical barrier against herbivory [Lobel and Ogden, 1981]. Selection for chemical defenses may be driven by intense herbivory, however there are many other factors contributing to this selection. It is for this reason, that the above toxicity assays were developed [Paul and Fenical, 1986].

The fact that these compounds which were isolated from green algae, were found to be highly bioactive, supports the hypothesis that they function as defense agents. Of the many diverse metabolites isolated from a wide spectrum of green, red and brown algae, few show the potent activity displayed by members of the Caulerpalean order.

Compounds possessing one or more aldehyde functional groups e.g. compound 5, are the most biologically active. Aldehyde groups, and especially  $\alpha$ ,  $\beta$ -unsaturated aldehydes, can react with biological molecules in several ways which result in enzyme deactivation and interference with normal metabolic functions.

Aldehydes can react with basic amino groups to form Schiff Bases (imines). Biological nucleophiles (e.g. amines, alcohols, sulfhydryl groups), can add to the  $\beta$ -carbon of the unsaturated aldehyde in a Michael Addition reaction. Very little is known however regarding the interactions of enol-acetate functionalities with biological molecules. Hydrolysis of the enol-acetates should occur *in vivo* to yield the bioactive aldehydes, but addition reactions directly to the unsaturated may also be possible [Paul and Fenical, 1986].

Another compound, isolated from *C.prolifera*, which was found to be active against the sea urchin *Lytechinus variegatus* and was responsible for feeding deterrent activities, was caulerpenyne (1) [McConnell *et al.*, 1982]. Similarly, the compound stypoldione (8) which is an ortho-quinone mono alcohol from the brown seaweed, *Stypopodium zonale*, was found to inhibit sea urchin embryo division in a way dependent-on concentration [O'Brien *et al.*, 1984].

(iv) Secondary Metabolites active against Crustaceans:

The genus *Laurencia* produces a number of compounds which confer a special ecological advantage on the alga, protecting them from excessive predation. *L.obtusa* produces sets of secondary metabolites which change from one location to the next. From a collection made in the Jamaican waters, three compounds were isolated, of which the major metabolite (+)- elatol (2) was the most important. This compound was found to be toxic to brine shrimp hatchlings, causing 78% death within 24 hrs. The two related minor metabolites of elatol both displayed similar activities to elatol, but at reduced levels [Brennan *et al.*, 1987].

(v) Secondary Metabolites active against Molluscs:

In studies by Paul and Fenical (1986), a gastropod feeding assay was carried out, which involved the feeding of four separate diets to juvenile conch (*Strombus costatus*). Of the compounds tested, halimedatrial (5) was found to cause 100% mortality within 6 days, whereas caulerpenyne (1) and udoteal (11) caused 50% mortality in the same period, after which the conch stopped grazing the treated algae and grazed exclusively on the diatoms adhering to the glass beakers. Although various other reasons have been given in the past for the high death rate, it is now believed, that the presence of these terpenoids are responsible [Paul and Fenical, 1986].

In another study involving gastropods, the chlorophyte *Anadyomene stellata* (Wulfen) C. Agardh which had previously been shown to cause tentacle withdrawal in these molluscs, showed activity within an aqueous extract which had been prepared. Most of the metabolites in green algae that have shown biological activity are in fact lipids, this unknown compound however has been hypothesized as being responsible for both the antibacterial activity shown, as well as the tentacle withdrawal. This hypothesis has not yet been proven [Hodgson, 1984].

### 1.3.9 Chemistry of Bioactive Compounds:

Due to the fact that many of the active compounds identified have halogen(s) in their structures, it was necessary to gain some background into the presence of these compounds and evaluate whether their presence influenced bioactivity or not. The red algae have gained particular interest, because they are known to synthesize a large diversity of halogenated organic compounds and also incorporate large

quantities of halides into their structures. These halogenated products are not involved in primary metabolic processes, but constitute the messengers of an exocrine system which evolved to provide selective environmental advantage to the algae [Fenical, 1975].

Members of the other groups of seaweeds also utilize halogens, but the red algae are unique in their utilization of all three halogens, particularly bromine (Br). Considering the concentration of halide ions in seawater,  $\text{Cl}^-$  19000  $\text{mg.l}^{-1}$ ,  $\text{Br}^-$  65  $\text{mg.l}^{-1}$  and  $\text{I}^-$  and  $\text{IO}_3^-$  0.06  $\text{mg.l}^{-1}$ , an unlimited supply of these elements exists. Even so, halide ions appear to selectively diffuse across the cell wall and accumulate for subsequent metabolic activities. Some uncertainty exists as to the state in which the halogens occur intracellularly, however from a study on the alga *Antithamnion plumula* (Ellis) Thuret, it appears, that the brominating entity is an enzyme-halogen complex. This complex is selective in its bromination of organic substrates and incapable of oxidizing iodide. Specialized cells, called vesicular or secretory cells, containing large amounts of halogens, are the site of halogenation in *Antithamnion* species. These specialized cells, however, are not a requirement of halogen metabolism, as they are absent in *Laurencia*, *Polysiphonia*, *Rhodomela* and *Plocamium* species, all of which metabolise halogens [Fenical, 1975].

A halogenating enzyme has been isolated from both red and green algae, which is known as bromoperoxidase. This enzyme is important as it is required for the assimilation and incorporation of Br into the alga. Studies have also shown, that an  $\text{I}^-$  oxidase system exists, which is capable of causing the iodination of the amino acid tyrosine, as well as the formation of  $\text{I}_2$ . It therefore appears, that selective halogenation occurs, whereby specific halogenating enzymes work on individual halogens [Fenical, 1975].

The structural features of the algal metabolic products generally suggests, that the reactive moiety is an enzyme bound halogen atom which is partially void of outer shell valence electrons i.e.  $\text{X}^+$  intermediate. This intermediate could be similar in structure to N-bromosuccinimide (NBS), and the reactive enzyme centre may be halogen substituted nitrogen atoms which are part of peptides (Fig. 2). This type of intermediate is known to react with halide ions to yield molecular halogen or to react with electron-rich organic compounds. When phenol is used as a substrate molecule, reaction with a  $\text{Br}^+$  source e.g. NBS, results in substitution at the ortho and para positions (Fig. 3). The bromophenols from red algae are suspected of occurring via these pathways.

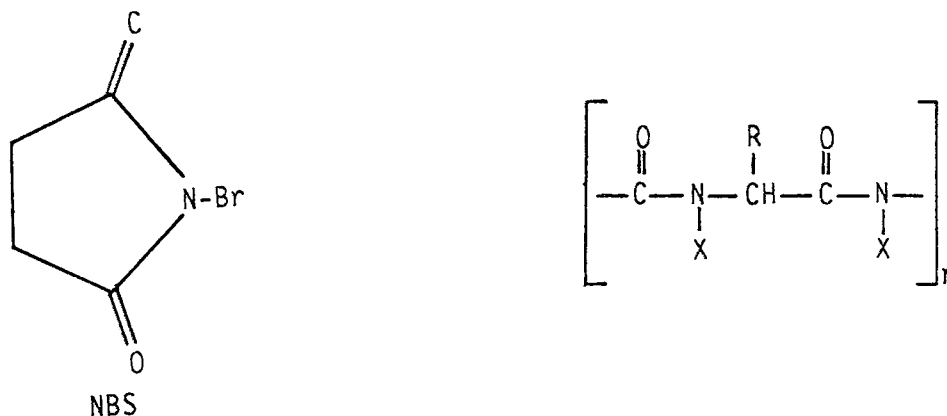


Fig. 2 The molecular structure of N-bromosuccinimide (NBS), and the possible active sites for halogenation in an algal enzyme [Fenical, 1975].

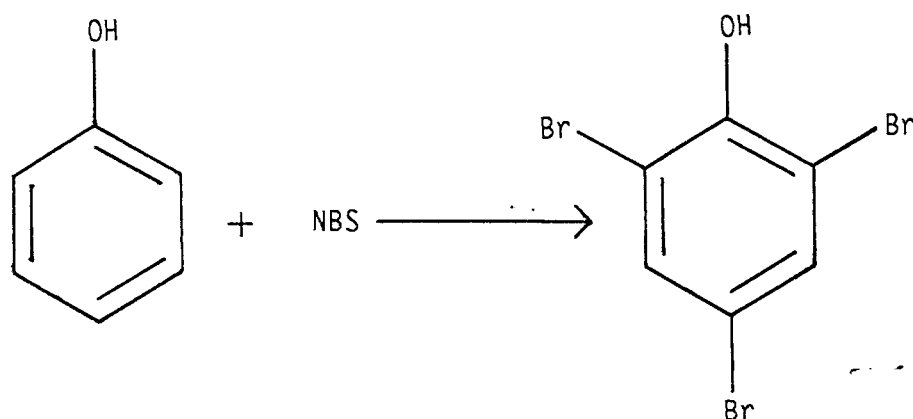


Fig 3 The reaction of N-bromosuccinimide (NBS) and phenol to give 2,4,6-tribromophenol [Fenical, 1975].

The majority of halogens in red algae occur in the ionic form,  $X^-$ . These are converted into organic halogen-containing substances and to a lesser extent to the molecular form. Few iodinated compounds are known. The iodinated tyrosine derivatives which were isolated after protein hydrolysis, cannot be considered as free cellular components.  $I^-$  however may be an essential element, as when it was limited in culture studies of *Polysiphonia urceolata* (Light.) Grev. and *Asparagopsis armata* Harvey, proper development was impeded [Fenical, 1975].

A large number of bromine and chlorine containing compounds exist in the red algae. The first red alga reported to have a high bromine content was that of *Rhodomela larix* C.Ag. Strong antibiotic properties were associated with this alga, and it was later shown, that it was the Br-containing phenols which were the biologically active components. Another example is that of *Asparagopsis taxiformis* (Delile) Trevisan which exhibited strong antimicrobial activity which was attributed to the accumulated brominated compounds present in the alga [Fenical, 1974, 1975].

The bromine-containing phenols occur primarily in the Rhodomelaceae (Ceramiales) and constitute 1-5% of the dry weight of the alga. The brominated compounds are probably derived biosynthetically from shikimic acid via a typical aromatic amino acid synthesis. It is probable, that bromination and side chain degradation occur after the construction of the aromatic ring. The bromophenols from *Laurencia*, laurinterol (3), isolaurinterol and laurenisol, are probably from a different origin, which involves the build-up of isoprene units in the biosynthesis of terpenes, via isopentenyl pyrophosphate.

Some compounds may originate from the dimerization of two compounds, (i), by condensation at the hydroxyl side of one monomer, leading to a substituted pattern i.e. avrainvilleol (ii). An alternative, is the same reaction at the other side, yielding 5'-hydroxyavrainvilleol (iii) (Fig. 4). Separate extractions of the blade, stipe and haptera of the plant *Avrainvillea nigricans* led to the finding that the secondary metabolites occur in greater quantities in the blade region than anywhere else. This observation suggests a defense role for these natural products [Colon *et al.*, 1987].

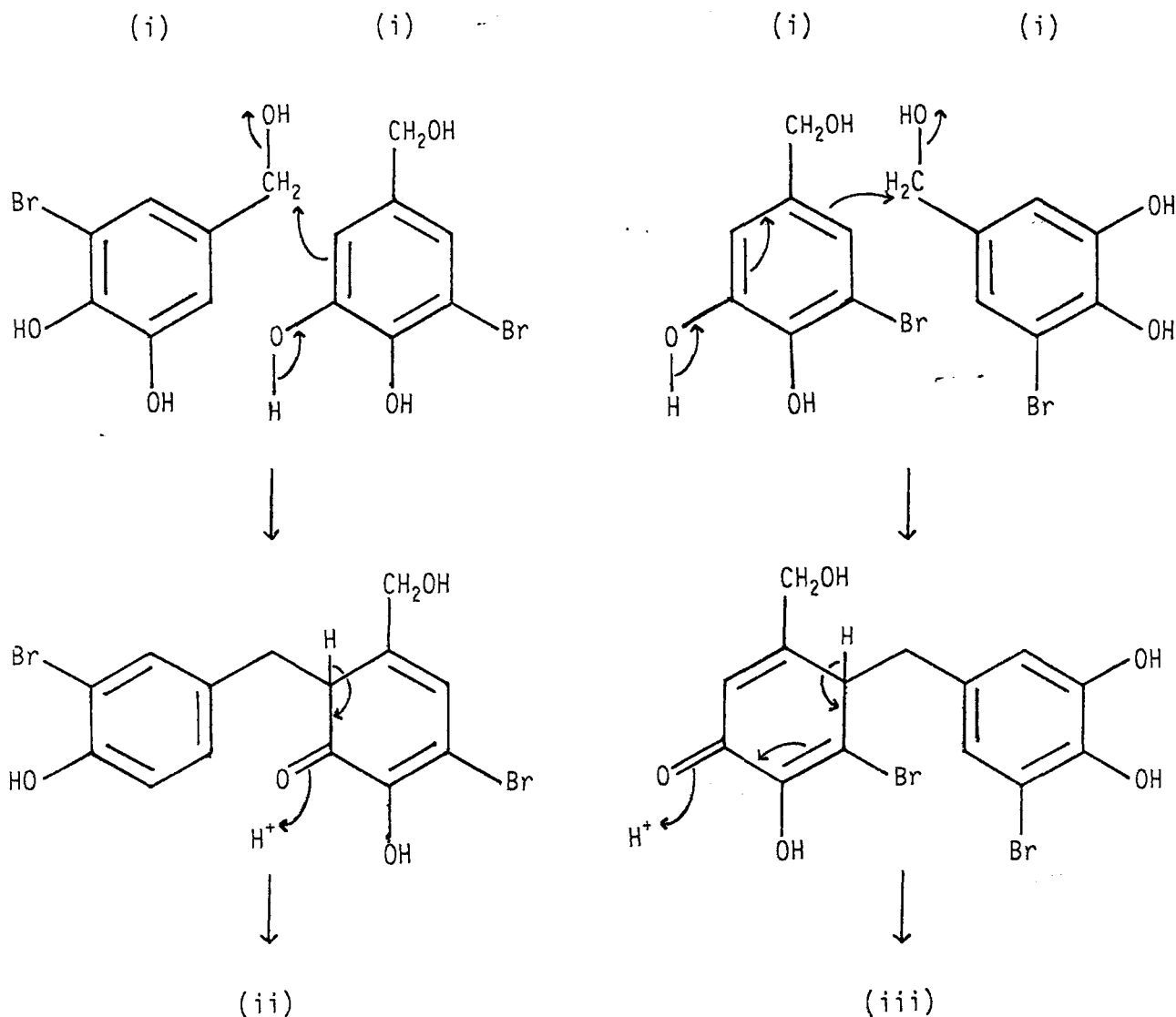
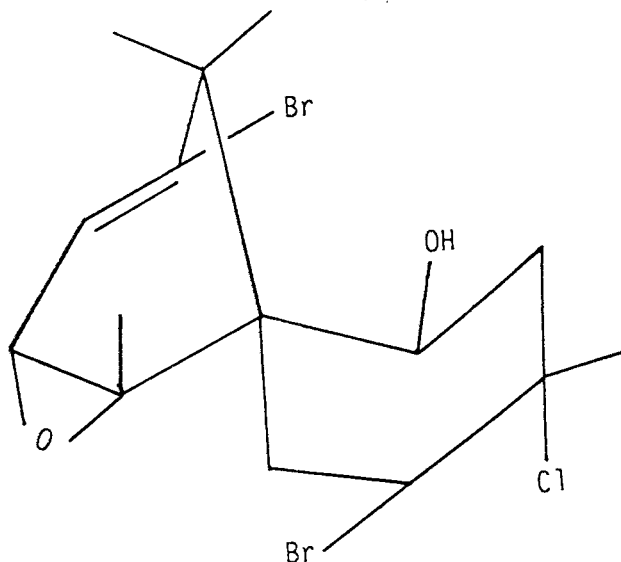


Fig. 4 The proposed biogenetic relationship of avrainvilleol (ii) and 5'-hydroxyavrainvilleol (iii) [Colon *et al.*, 1987].

In a study by Sims *et al.* (1975), five compounds with potent antimicrobial activity were found. Of these, three of particular interest include laurinterol (3), cycloeudesmol (4), and debromolaurinterol (6). Of these compounds, only laurinterol contained Br. A direct comparison could therefore be carried out on the compounds laurinterol and debromolaurinterol to give some indication of the amount of antimicrobial activity contributed by the halogen, bromine, specifically; as the ring structure is identical. In this study, laurinterol showed only slightly better activity than debromolaurinterol, suggesting only slight influence by the Br atom. The compound cycloeudesmol, containing no Br, showed excellent activity. This compound however has a different ring structure which imparts bioactive properties [Sims *et al.*, 1975]. The actual ring structure may therefore be more important than the halogen with regards to bioactive potential.

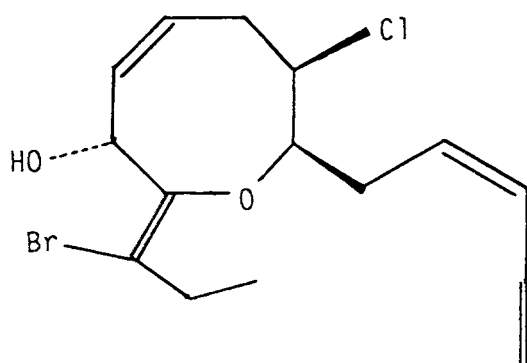
Terpenes are common in three genera of red algae only, namely *Laurencia*, *Plocamium* and *Desmia*. The most commonly occurring halogenated metabolites are the sesquiterpenoids ( $C_{15}$ ) which are composed

of three isoprene units. Of the brominated sesqui- and diterpenes ( $C_{20}$ ) discovered, all occur in various *Laurencia* species. Members of this genus have the ability to synthesize structurally elaborate halogenated natural products with different carbon skeletons. Nine different terpene skeletons have been produced, of which the chamigrene carbon system, found in *Laurencia* is most common. Pre-pacifenol (12) has the above structure, and was found by Sims *et al.* (1975) to exhibit potent antimicrobial activity.



Compound 12 Pre-pacifenol, has a chamigrene carbon skeleton, and exhibits potent antimicrobial activity [Sims *et al.*, 1975].

*Laurencia* species are also capable of synthesizing halogen-containing products from fatty acids which are derived via acetate biosynthesis. The common feature of these non-terpenoid compounds, is the 15-C atoms and a conjugated pentenyne group e.g. chondriol (13). The pentenyne group is unique and has been found to be responsible for cytotoxicity and potent antimicrobial reactions.



Compound 13 Chondriol, a non-terpenoid with a conjugated pentenyne group which is responsible for the cytotoxicity, and potent antimicrobial activity [Sims *et al.*, 1975].

Members of the families Caulerpaceae and Udoteaceae, which are closely related, have been shown to produce metabolites which have a unique functional group, E,E-1,4-diacetoxybutadiene. It is this functional group which is believed to be responsible for the biological activity displayed by many of the diterpenoid compounds which have been isolated. The majority of the bioactive secondary metabolites found in Caulerpaceae are sesquiterpenoid ( $C_{15}$ ) or diterpenoid ( $C_{20}$ ) compounds, and although produced

in high concentrations, these compounds are not easily isolated due to their instability and reactive chemical natures.

The diversity of metabolites isolated from the order Caulerpales is great, but even so, there are common unifying features. The linear or acyclic form of many of the terpenoids is one feature, with the conjugated bis-enol functional group being another. Until its recent discovery in algae, this functional group was unknown in natural products, and it is this which is responsible for the high biological activity. This group is referred to as a 'masked' or acetylated dialdehyde constellation [Paul and Fenical, 1986, 1987].

It is these studies that have confirmed the reasons for the Caulerpalean algae being as abundant as they are. It is not entirely due to calcification, but due to the production of unique secondary metabolites containing aldehyde or enol acetate functional groups, as mentioned above. Results obtained support the hypothesis that these secondary metabolites function as defense against both predators and pathogens. In fact all the compounds tested were found to be highly bioactive [Paul, 1985]. The aldehyde groups, and especially  $\alpha,\beta$ -unsaturated aldehydes react with biological molecules, resulting in deactivation of enzymes and interference with normal metabolic functions.

These molecules clearly shown great promise in new drug development due to their potent biological effects. To evaluate these drugs for pharmaceutical use, far more comprehensive pharmacological studies must be carried out, to determine the potential activities and therapeutic applications of these terpenoids [Fenical and Paul, 1984].

Crude extracts of brown algae (Phaeophyceae) have been shown to exhibit *in vitro* activity against Gram positive bacteria. This can be explained by the presence of phenols in the extracts. The brown algal cells characteristically contain refractile bodies called physodes. These originate in the plastids, and contain tannins which are phenolic and polyphenolic compounds which are active defouling agents. These phenolics are toxic, and act by binding and inactivating proteins [Baker, 1984].

In conclusion, it appears from the above review that:

- (i)  $I^-$  is required for the development of algae and has no antibiotic influence
- (ii)  $Br^-$  containing phenols exhibit biological activity; the phenol appears to be the active component
- (iii) The experiment by Sims *et al.* (1975) using laurinterol and debromolaurinterol proved the limited influence of Br as an antimicrobial agent
- (iv) The activity displayed by *Asparagopsis taxiformis* was attributed to brominated compounds, however the type of compound was not specified.
- (v) The actual ring structure appears to be of importance in bioactive compounds
- (vi) Specific functional groups impart exceptional biological activity, these react with proteins etc. and so inactivate them.

Table 1.3.1 A summary of some of the active compounds isolated from seaweeds

| Compound                             | Cpd. No. | Activity                                                    | Reference                                                                                           |
|--------------------------------------|----------|-------------------------------------------------------------|-----------------------------------------------------------------------------------------------------|
| Caulerpenyne                         | 1        | antibacterial<br>ichthyotoxic<br>antimolluscan              | Hodgson, 1984<br>Lobel and Ogden, 1981<br>Paul and Fenical, 1986                                    |
| Elatol                               | 2        | antimicrobial<br>brine shrimp toxic                         | Ballantine <i>et al.</i> , 1987<br>Brennan <i>et al.</i> , 1987                                     |
| Laurinterol                          | 3        | antimicrobial                                               | Sims <i>et al.</i> , 1975                                                                           |
| Cycloeudesmol                        | 4        | antimicrobial                                               | Sims <i>et al.</i> , 1975                                                                           |
| Halimedatriol                        | 5        | antimicrobial<br>ichthyotoxic<br>antimolluscan<br>cytotoxic | Fenical and Paul, 1984<br>Lobel and Ogden, 1981<br>Paul and Fenical, 1986<br>Fenical and Paul, 1984 |
| Debromolaurinterol                   | 6        | antimicrobial                                               | Sims <i>et al.</i> , 1975                                                                           |
| 3-bromo-4, 5-dihydroxybenzyl alcohol | 7        | anticancer                                                  | Hodgson, 1984                                                                                       |
| Stypoldione                          | 8        | anticancer<br>echinoderm toxic                              | O'Brien <i>et al.</i> , 1984                                                                        |
| Thyrsiferol                          | 9        | antiviral                                                   | Sakemi <i>et al.</i> , 1986                                                                         |
| Venustatriol                         | 10       | antiviral                                                   | Sakemi <i>et al.</i> , 1986                                                                         |
| Udoteal                              | 11       | ichthyotoxic<br>antimolluscan                               | Lobel and Ogden, 1981<br>Paul and Fenical, 1986                                                     |
| Pre-pacifenol                        | 12       | antimicrobial                                               | Sims <i>et al.</i> , 1975                                                                           |
| Chondriol                            | 13       | antimicrobial<br>cytotoxic                                  | Sims <i>et al.</i> , 1975                                                                           |

#### 1.3.10 Ecological Role of Bioactive Agents in Algae:

Antibiosis is the detrimental effect that one organism has on another via some chemical compound (antibiotic). Antibiosis by seaweeds is usually directed towards either epiphytes or grazers. The release of secondary metabolites, however, are not always used as defense or protective agents for the algae, they are sometimes essential ingredients in the chain of reproductive events.

Many aromatic compounds isolated from seaweeds, which have displayed antibiotic properties, have not been studied to determine the true function of the compound within the algal thallus. One example of an aromatic compound playing an important role in the life-cycle of the alga is that of the kelp *Laminaria*. The life-cycle of this alga has an alternation of generations in which motile haploid zoospores are produced in the sporangia. Small filamentous gametophytes grow from the zoospore, which in turn produce the motile sperm and immotile egg. It is at this stage that the mature female gametes produce a simple aromatic compound which serves to attract the male gamete or sperm, to ensure fertilization. This aromatic compound may, or may not have antibiotic properties, yet it is produced by the alga for a specific yet essential function within the life-cycle.

Further investigations into the reasons for the production of the compounds found to have antibiotic properties may yield similar results. The main emphasis world-wide however, is for the discovery of new metabolites with biomedicinal properties. Whether they are produced for a specific purpose or not is of no consequence and as a result of this, very little research has been carried out to ascertain the exact reasons for their production [Paul and Fenical, 1986].

Many of the active compounds identified, have halogens in their structures. The bromophenol, lanasol, was found to be toxic to microalgae grown in cell culture and caused cell lysis in a number of algal species, this suggests that this compound could deter epiphytic algal encrustation. The sulfate salts of these phenols however, showed no anti-algal properties and were in fact mild growth stimulants. It is thought, that the bromophenols occur naturally in the sulfate form and hence their role in preventing epiphytic growth is questionable. Sulfates however, are easily hydrolysed to active phenols, this could occur on the thallus surface as the sulfates are exposed to sea water.

Halogen-containing metabolites also show great promise in the biomedicinal field, however rather than harvesting large quantities of marine flora, these natural products will serve as model systems for laboratory synthesis of new medicines and biocides. Because these new substances will be based on natural products, they should have the added advantage of being biodegradable.

As it has been recognized that marine organisms produce large amounts of halogenated compounds, the effect of pesticide pollution on the oceans could have great impact. Many industrial biocides are chlorinated compounds similar to those produced by algae. If these are incorporated into the natural biodegradation process, no problems should arise, however if the natural halogen-containing compounds are the messengers of sophisticated biological communications, the danger does exist, that the industrial compounds could mimic these processes and so upset nature's balance [Fenical, 1975].

The phenolic and polyphenolic compounds produced by the brown algae which act as active defouling agents are toxic, because they act by binding and inactivating proteins. The *Sargassum* species in the Sargasso Sea produce tannins; this production correlates with the number of physodes in the cells. Only the tips of the algae in the centre of the Sargasso sea are free of epiphytes, this being due to activity being concentrated in the growing tips. The growth and establishment of barnacles (*Balanus balanoides*) and mussels (*Mytilus edulis*) in tidal pools is suppressed by the domination of *Ralfsia*, but not by *Hildenbrandia*. The presence of phenolic compounds appears to be responsible for this suppression as well. The brown algae have not only attracted attention because of these products, but also because of

the world-wide distribution of some families, and their ability to survive in areas of intense herbivory with no mechanical defenses. One family of such ability is that of Dictyotaceae which has colonized the infralittoral zone world-wide. This strongly suggests the presence of secondary metabolites for chemical defense.

The diterpene acetoxycrenulide was first isolated from the alga *Dictyota crenulata*, and later from the sea hare *Aplysia vaccaria*. A relationship exists which is of interest, because this diterpene possesses ichthyotoxic properties and so plays a fundamental role in the defense strategy of the mollusc.

The above examples give some indication of the complexity of the ecological interactions that take place in tropical marine systems. It is also possible, that these same diterpenes are fagostimulants or attractants for the specific carnivorous predators of the sea hare. If this is the case, the defense compound of the alga may be found further down the food chain, several links away from the actual producer [Kelecom and Teixeira, 1986].

Chemical deterrents are produced by large numbers of algae, some species of the brown algae, *Desmarestia* for example produces high sulphuric acid concentrations in the cells, some have a vacuolar pH of less than one. The red and green algae are also known to produce antibiotic chemicals. Udoteal (11), the diterpenoid lipid, obtained from *Udotea flabellum* (Ellis and Solander) Lamouroux deters herbivorous fish, and the brominated phenol isolated from the related genus *Avrainvillea* plays the same role. In preference trials, sea urchins have been found to show slight avoidance of *Agarum*, and distinct avoidance of the red alga *Opuntella*; the compounds responsible for this behaviour have not been isolated. It is evident, that the interactions of seaweeds, grazers and the predators of the herbivores require much more attention for them to be fully understood [Lobban *et al.*, 1985].

From this brief report, it is evident, that a limited amount of work has been carried out on the ecological role of bioactive agents in algae. This is a very interesting aspect of secondary metabolite production and function, which requires a lot more work.

## CHAPTER II

### THE EFFECT OF DIFFERENT EXTRACTION SOLVENTS ON SEAWEED BIOACTIVITY.

#### 2.1 Introduction:

Various solvents have been used in previous studies to extract antimicrobial, antiviral and anticancer agents from seaweeds, microalgae and plants. These are summarized in Table 2.1.1.

Table 2.1.1 The solvents used for the extraction of bioactive compounds

| Solvent                                  | Use                                                                                                                | Reference                                                                                                                |
|------------------------------------------|--------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------|
| Water                                    | anticancer<br>anti-HSV-I<br>Toxicity testing using mice                                                            | Hodgson, 1984<br>Stein and Borden, 1984<br>Vidal <i>et al.</i> , 1984                                                    |
| Methanol                                 | antimicrobial and anticancer<br>antimicrobial and anticancer toxicity testing using mice.<br>antibacterial         | Hodgson, 1984<br>Stein and Borden, 1984<br>Vidal <i>et al.</i> , 1984<br>Fabregas and Veiga, 1984                        |
| Methanol/Toluene (3:1)                   | antimicrobial and antiviral<br>anticancer<br>anticancer                                                            | Caccamese <i>et al.</i> , 1984<br>Kobayashi <i>et al.</i> , 1986<br>Kobayashi <i>et al.</i> , 1988                       |
| Ethanol                                  | antiviral<br>lipid-soluble compounds<br>antitumour<br>antitumour                                                   | Ehresmann <i>et al.</i> , 1977<br>Littler and Littler, 1985<br>Hayashi <i>et al.</i> , 1987<br>Hori <i>et al.</i> , 1987 |
| Ethanol, then dichloromethane            | chemical defense compounds                                                                                         | Paul <i>et al.</i> , 1987                                                                                                |
| Dichloromethane                          | chemical defense compounds                                                                                         | Paul and Fenical, 1986                                                                                                   |
| Chloroform                               | lipid-soluble compounds<br>antimicrobial and anticancer<br>-chloroform causes degradation of some natural products | Littler and Littler, 1985<br>Hodgson, 1984<br>Colon <i>et al.</i> , 1987                                                 |
| Dichloromethane\Methanol (1:1)           | antifungal                                                                                                         | Brennan <i>et al.</i> , 1987                                                                                             |
| (2:1)                                    | antimicrobial fatty acids<br>antimicrobial lipid-soluble compounds                                                 | Lopez and Gerwick, 1987<br>Ballantine <i>et al.</i> , 1987                                                               |
| Diethyl ether                            | lipid-soluble compounds<br>antimicrobial and anticancer                                                            | Littler and Littler, 1985<br>Colon <i>et al.</i> , 1987                                                                  |
| Ethyl acetate                            | antimicrobial and anticancer                                                                                       | Colon <i>et al.</i> , 1987                                                                                               |
| Methanol, then Chloroform\methanol (2:1) | anti-Gram positive bacteria<br>ichthyotoxicity                                                                     | Schlenk and Gerwick, 1987                                                                                                |
| Acetone                                  | anti-VSV and anti-HSV-I<br>antimicrobial<br>antibacterial<br>antibacterial                                         | Sakemi <i>et al.</i> , 1986<br>Hornsey and Hide, 1976 (a)<br>Sultanbawa <i>et al.</i> , 1987<br>Fabregas and Veiga, 1984 |

|                                   |                                      |                                                                    |
|-----------------------------------|--------------------------------------|--------------------------------------------------------------------|
| Ether                             | toxicity testing using mice          | Vidal <i>et al.</i> , 1984                                         |
| Methylene chloride                | toxicity testing using mice          | Vidal <i>et al.</i> , 1984                                         |
| None, only crushed plant material | antifungal essential oils            | Janssen <i>et al.</i> , 1987                                       |
| methanol, then CHCl <sub>3</sub>  | hepatotoxic to mammals<br>antitumour | Wassel <i>et al.</i> , 1987 (a)<br>Wassel <i>et al.</i> , 1987 (a) |

The objective of the present study using different solvents was to determine which solvent could extract the biologically active secondary metabolites most effectively. Various solvents with differing polarities were therefore used in this study. The solvent giving the best results would be used for all subsequent extractions. The aim therefore was to assess the solvents used by determining the effect of the different seaweed solvent extracts on the test microorganisms. Because of the magnitude of the entire project, the use of more than one solvent would have been impracticable.

## 2.2 Materials and Methods:

The nine algae which were collected from the rocky outcrop known as the First Sister, Riet River at low tide on the 5 March 1987 are listed in Table 2.2.1. The algae were placed in plastic bags, drained of all excess seawater and placed on ice for transportation back to the laboratory. It was essential to chill the seaweeds as quickly as possible, as chilling reduces the denaturation of enzymes etc. which starts as soon as the seaweeds are harvested. Once identified, the seaweeds were stored at -20°C. When required, they were thawed, cleaned of epiphytes and other extraneous material and rinsed quickly in distilled water. Alga (10g wet weight) was extracted in 50 ml of solvent. The solvents used are shown in Table 2.2.2.

Table 2.2.1. Algae collected from the First Sister, Riet River, on 5 March 1987

| Group         | Alga                                                                                                                                                      |
|---------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------|
| Rhodophyceae  | <i>Amphiroa anceps</i> (Lamarck) Decaisne<br><i>Spyridia cuppressina</i> (Harvey) Kuetzing<br><i>Hypnea spicifera</i> (Suhr) Harvey                       |
| Phaeophyceae  | <i>Sargassum heterophyllum</i> (Turner) C.Agardh<br><i>Zonaria subarticulata</i> (Lamouroux)<br>Papenfuss<br><i>Ecklonia biruncinata</i> (Bory) Papenfuss |
| Chlorophyceae | <i>Ulva rigida</i> C.Agardh<br><i>Caulerpa filiformis</i> (Suhr) Hering<br><i>Codium duthieae</i> Silva                                                   |

Table 2.2.2 Solvents used for antimicrobial testing

| Solvent                         |
|---------------------------------|
| Water                           |
| Methanol/toluene (3:1)          |
| Ethanol                         |
| Acetone                         |
| n-Butanol                       |
| Ether                           |
| hexane, ethyl acetate, methanol |

The hexane ethyl acetate, methanol extraction involved using a reflux condenser. Hexane, then ethyl acetate, and finally methanol was used, but only the methanolic extract was tested against the microorganisms.

The algae were homogenised on ice for 2 minutes using a Du Pont Sorvall Omni-Mixer 17106. After this the homogenates were filtered through Whatman No. 1 paper to remove the particulate matter which included cell debris, cellulose, algin, fibre etc. The samples were frozen again at -20°C, then filtered under vacuum, in the frozen state, for a second time using a buchner funnel. The second filtration served to remove excess water from the non-polar samples as the ice crystals did not pass through the filter.

The filtered samples were then dried. The water extracts were freeze dried, and the remainder dried using rotary evaporation. The drying process was important, as many of the solvents may have been toxic to the test organisms. The dried extracts were resuspended in 2 ml phosphate buffer (0,1 M pH 7,5). At this stage, the extracts were ready for testing against the microorganisms.

In this work, the microorganisms were grown in nutrient or Wickerham's broth overnight. Prepared nutrient agar or Wickerham's agar plates were seeded with 200 µl of this overnight culture and spread using a sterilized glass rod, to produce an even lawn of bacteria. The microorganisms used are shown in Table 2.2.3.

Table 2.2.3. Test organisms

|          |               |                                                                                                    |
|----------|---------------|----------------------------------------------------------------------------------------------------|
| Bacteria | Gram negative | <i>Escherichia coli</i> (Ec)<br><i>Pseudomonas aeruginosa</i> (Pa)                                 |
|          | Gram positive | <i>Bacillus subtilis</i> (bs)<br><i>Staphylococcus aureus</i> (Sa)<br><i>Micrococcus sp.</i> (Msp) |
| Yeast    |               | <i>Candida albicans</i> (Ca)                                                                       |

Two methods were used to test the activity of the seaweed extracts. The agar well-diffusion method [Hornsey and Hide, 1976] and the paper disc method [Caccamese *et al.*, 1980]. The agar well diffusion

method involved punching "wells" in the seeded agar using the back of a sterilized pasteur pipette. These plugs were then removed and the wells filled with the sterile, filtered algal extracts. The plates were incubated at 37°C for 24-48 hours after which the diameter of the zones of inhibition around the wells were measured. The paper disc method involved punching paper discs of constant diameter out of Whatman No. 1 filter paper. These discs were sterilized by placing in a sealed glass bottle and autoclaving at 120°C for 20 minutes. The discs were then dipped into the algal extracts, dried at 37°C, then placed on newly seeded agar plates. The plates were incubated as above. All tests were carried out in duplicate and under strictly sterile conditions.

During the 24-48 hour growth period, one problem arose; this was the growth of contaminants in the extracts. This could have been introduced into the extract solutions during the extractions process. This interfered with the growth of the chosen test organism. Various methods were tried to determine which was the most effective in contaminant eradication. It must be kept in mind, that in the practice of sterilization, the microbial population to be destroyed, was invariably a mixed one. As microorganisms vary in their resistance to destruction, the death rate of the most resistant members of the mixed population are of importance. Heat sterilization is the most commonly used lethal agent, with autoclaving being an example of heat sterilization at high temperature and pressure. The autoclave operates at a steam pressure of 1 bar above atmospheric pressure, which corresponds to a temperature of 120°C. Sterilization results after exposure to these conditions for a period of 20 minutes. This method of sterilization however is not suitable where proteins are present, as protein denaturation occurs at 60°C.

Ultraviolet (UV) irradiation is another recognized method of sterilization. This method however requires more time, up to 24 hours of constant irradiation, to be effective. It is not possible, for UV light to pass through glass, with the result that this method could not be used to sterilize solutions in bottles.

The principle laboratory technique for the sterilization of heat-labile materials is filtration through complex filters with pore sizes capable of retaining microorganisms e.g. 0,2 or 0,45 µm diameter pores. The complexity of these filters results in the microorganisms either being retained by the small pore size, or by absorption to the walls of the pores. Bacteria have a wide size range, but are too large to pass through 0,2 or 0,45 µm filters. Viruses on the other hand are far smaller and are therefore not necessarily retained by filtration. It is therefore not possible to be certain that filtration that has rendered a solution bacterium-free, will also render it virus-free.

Sonication can also be used as a method of sterilization. Small amounts of the extracts were sonicated at maximum amplitude for 2 seconds in Eppendorf tubes, then tested.

To enhance the inhibitory zones to make measurement easier, the virtually colourless dye 2,3,5-triphenyltetrazolium chloride (TTC) was added to nutrient agar, this together with bacteria was used as an overlay on the agar [Williams and Wilson, 1984]. The "wells" were punched through both layers of agar, then filled with extract. This dye produces an intense red colour on reduction, due to the production of formazan.

## 2.3 Results and Discussion:

### Well diffusion vs Paper disc technique:

From the antimicrobial results obtained using the agar well diffusion and paper disc techniques, it was decided, that the paper disc technique would be used for all further studies.

### Sterilization:

Of the sterilization methods tried, filtration through 0,45 µm filters proved to be the easiest and quickest method. This method had no detrimental effects on the bioactivity of the extracts.

### Enhancing technique:

The use of 2,3,5 triphenyltetrazolium chloride as an enhancing agent proved to be unsuccessful. The reason for this being that on reduction the colourless dye became an intense red colour, however many of the brown algae (Phaeophyceae) produce phenols which are dark brown in colour. These phenols migrated through the agar and in so doing prevented clear resolution of the zones of inhibition.

### Solvent testing:

The results of the solvent testing are shown in Table 2.3.1.

Table 2.3.1. The antimicrobial activity of selected seaweeds extracted in different solvents.

Table 2.3.1.

| Seaweed species            | Microorganism | Solvents |         |         |       |         |                      |                                       |
|----------------------------|---------------|----------|---------|---------|-------|---------|----------------------|---------------------------------------|
|                            |               | water    | ethanol | acetone | ether | butanol | methanol/<br>toluene | hexane, ethyl<br>acetate,<br>methanol |
| Rhodophyta                 |               |          |         |         |       |         |                      |                                       |
| <i>Amphiroa anceps</i>     | Sa            | 0        | 0       | 0       | 0     | 0       | 0                    | 0                                     |
|                            | Bs            | 0        | 0       | 0       | 0     | 0       | 0                    | 0                                     |
|                            | Msp           | 0        | 0       | 0       | 0     | 0       | 0                    | 0                                     |
|                            | Ec            | 0        | 0       | 0       | 0     | 0       | 0                    | 0                                     |
|                            | Pa            | 0        | 0       | 0       | 0     | 0       | 0                    | 0                                     |
|                            | Ca            | 0        | 0       | 0       | 0     | 0       | 0                    | 0                                     |
| <i>Spyridia cupressina</i> | Sa            | 0        | 0       | 0       | 0     | 0       | 0                    | 0                                     |
|                            | Bs            | 0        | 0       | 0       | 0     | 0       | 0                    | 0                                     |
|                            | Msp           | 0        | 0       | 0       | 0     | 0       | 0                    | 0                                     |
|                            | Ec            | 0        | 0       | 0       | 0     | 0       | 0                    | 0                                     |
|                            | Pa            | 0        | 0       | 0       | 0     | 0       | 0                    | 0                                     |
|                            | Ca            | 0        | 0       | 0       | 0     | 0       | 0                    | 0                                     |
| <i>Hypnea spicifera</i>    | Sa            | 3.5      | 0       | 0       | 0     | 0       | 0                    | 0                                     |
|                            | Bs            | 0        | 0       | 0       | 0     | 0       | 0                    | 0                                     |
|                            | Msp           | 0        | 0       | 0       | 0     | 0       | 0                    | 0                                     |
|                            | Ec            | 0        | 0       | 0       | 0     | 0       | 0                    | 0                                     |
|                            | Pa            | 0        | 0       | 0       | 0     | 0       | 0                    | 0                                     |
|                            | Ca            | 0        | 0       | 0       | 0     | 0       | 0                    | 0                                     |

|                                |     |     |     |     |   |     |     |     |
|--------------------------------|-----|-----|-----|-----|---|-----|-----|-----|
| Phaeophyta                     |     |     |     |     |   |     |     |     |
| <i>Zonaria subarticulata</i>   | Sa  | 3.8 | 6.9 | 7   | 0 | 0   | 6   | 8.7 |
|                                | Bs  | 0   | 0   | 0   | 0 | 0   | 0   | 0   |
|                                | Msp | 3.8 | 6.4 | 4.5 | 0 | 3.9 | 4.4 | 2.1 |
|                                | Ec  | 0   | 0   | 0   | 0 | 0   | 0   | 0   |
|                                | Pa  | 0   | 0   | 0   | 0 | 0   | 0   | 2.3 |
|                                | Ca  | 0   | 0   | 0   | 0 | 0   | 0   | 0   |
| <i>Ecklonia biruncinata</i>    | Sa  | 0   | 0   | 0   | 0 | 0   | 0   | 5.1 |
|                                | Bs  | 0   | 0   | 0   | 0 | 0   | 0   | 0   |
|                                | Msp | 0   | 4.8 | 4.5 | 0 | 0   | 6   | 5.8 |
|                                | Ec  | 0   | 0   | 0   | 0 | 0   | 0   | 0   |
|                                | Pa  | 0   | 0   | 0   | 0 | 0   | 0   | 4.1 |
|                                | Ca  | 0   | 0   | 0   | 0 | 0   | 0   | 0   |
| <i>Sargassum heterophyllum</i> | Sa  | 0   | 0   | 0   | 0 | 0   | 0   | 2.9 |
|                                | Bs  | 0   | 0   | 0   | 0 | 0   | 0   | 0   |
|                                | Msp | 0   | 0   | 0   | 0 | 0   | 0   | 0   |
|                                | Ec  | 0   | 0   | 0   | 0 | 0   | 0   | 0   |
|                                | Pa  | 0   | 0   | 0   | 0 | 0   | 0   | 0   |
|                                | Ca  | 0   | 0   | 0   | 0 | 0   | 0   | 0   |
| Chlorophyta                    |     |     |     |     |   |     |     |     |
| <i>Ulva rigida</i>             | Sa  | 0   | 0   | 0   | 0 | 0   | 0   | 0   |
|                                | Bs  | 0   | 0   | 0   | 0 | 0   | 0   | 0   |
|                                | Msp | 0   | 0   | 0   | 0 | 0   | 0   | 0   |
|                                | Ec  | 0   | 0   | 0   | 0 | 0   | 0   | 0   |
|                                | Pa  | 0   | 0   | 0   | 0 | 0   | 0   | 0   |
|                                | Ca  | 0   | 0   | 0   | 0 | 0   | 0   | 0   |
| <i>Caulerpa filiformis</i>     | Sa  | 0   | 0   | 0   | 0 | 0   | 0   | 0   |
|                                | Bs  | 0   | 0   | 0   | 0 | 0   | 0   | 0   |
|                                | Msp | 0   | 0   | 0   | 0 | 0   | 0   | 0   |
|                                | Ec  | 0   | 0   | 0   | 0 | 0   | 0   | 0   |
|                                | Pa  | 0   | 0   | 0   | 0 | 0   | 0   | 0   |
|                                | Ca  | 0   | 0   | 0   | 0 | 0   | 0   | 0   |
| <i>Codium duthieae</i>         | Sa  | 0   | 0   | 0   | 0 | 0   | 0   | 0   |
|                                | Bs  | 0   | 0   | 0   | 0 | 0   | 0   | 0   |
|                                | Msp | 0   | 0   | 0   | 0 | 0   | 0   | 0   |
|                                | Ec  | 0   | 0   | 0   | 0 | 0   | 0   | 0   |
|                                | Pa  | 0   | 0   | 0   | 0 | 0   | 0   | 0   |
|                                | Ca  | 0   | 0   | 0   | 0 | 0   | 0   | 0   |

Three variables are present, these being:

- (i) solvents
- (ii) microorganisms
- (iii) seaweeds

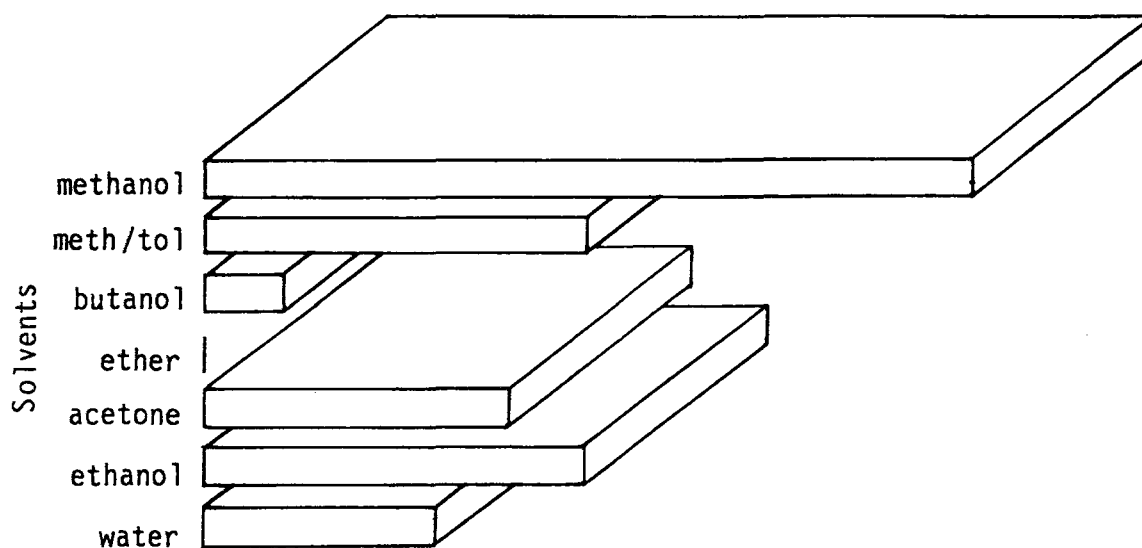
It was therefore necessary when analysing the above data to pool two sets of data. To assess the solvent data, the microorganism and seaweed data were pooled to produce Table 2.3.2. which follows.

Table 2.3.2. A comparison of the effects of different solvents on seaweed bioactivity.

| Solvent                         | Inhibition events<br>(possible 54) | Solvent Potency<br>Index (SPI) |
|---------------------------------|------------------------------------|--------------------------------|
| water                           | 3                                  | 3                              |
| ethanol                         | 3                                  | 5                              |
| acetone                         | 3                                  | 4                              |
| ether                           | 0                                  | 0                              |
| butanol                         | 1                                  | 1                              |
| methanol/toluene 3:1            | 3                                  | 5                              |
| hexane, ethyl acetate, methanol | 7                                  | 10                             |

(microorganism and seaweed data pooled)

Potency Index    1 = (zone of inhibition 7 – 10 mm)  
                       2 = (zone of inhibition 11 – 15 mm)  
                       3 = (zone of inhibition 16 – 20 mm)



Potency of Extract Inhibition Index in mm.

Fig. 5 A graphic representation of the different solvent/seaweed bioactivity data (Table 2.3.2).

In Table 2.3.2., the methanolic extract is shown to have the highest number of inhibition events, and a higher SPI. This shows that of the 7 inhibition events recorded, some have a greater potency than 1. Fig. 5 shows, that the methanolic extract is undoubtedly the most effective ( $P < 0.001$ ), and that the different solvents do not extract the bioactive secondary metabolites from seaweeds equally.

After methanol, the ethanolic and methanol/toluene extracts showed high activities, with acetone being close behind them. The polarities of these solvents, as reflected by their dielectric constants are methanol 33,6, ethanol 24,3 and acetone 20,7. Those solvents showing less activity than acetone include water 78,3, butanol 17,8 and ether 4,34. The three top solvents do not differ greatly in their polarities, this would suggest the presence of a single active compound which was extracted by all three solvents. If a single compound was present, varying intensities of activity would be expected depending on the polarity. Maximum activity would be expected at the optimum polarity for that particular compound, with reducing degrees of activity as the polarity shifts from the ideal value. This is evident from Fig. 5, as the polarity of water is much higher, and butanol and ether much lower than methanol.

As a result of the above tests, it was decided to use methanol as the solvent for all subsequent extractions, as it generally appears that the more polar solvents in the dielectric constant range of 20,7 – 33,6 gave the highest positive antimicrobial activity. The advantages of using methanol are that it is miscible with water, is relatively inexpensive and is suitable for rapid concentration of extracts with its low boiling point of 65,4°C.

## CHAPTER III

### THE SEASONAL ANTIMICROBIAL ACTIVITY OF SEAWEED EXTRACTS.

#### 3.1 Introduction:

Hornsey and Hide (1976(a)) did extensive studies on seasonal variations in antibiotic production from seaweeds. From these studies, it was found that four major patterns of production exist, these are shown in Table 3.1.1.

Table 3.1.1 The seasonal patterns of antimicrobial production from seaweeds [Hornsey and Hide, 1976(a)].

| Type                   | Time of year                           | Example                                                                                                                                    |
|------------------------|----------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------|
| 1. <i>Polysiphonia</i> | production uniform throughout the year | <i>Polysiphonia lanosa</i>                                                                                                                 |
| 2. <i>Laminaria</i>    | winter peak of production              | <i>Chondrus crispus</i><br><i>Laurencia pinnatifida</i><br><i>Ulva lactuca</i><br><i>Laminaria digitata</i><br><i>Laminaria saccharina</i> |
| 3. <i>Dictyota</i>     | summer peak of production              | <i>Dictyota dichotoma</i><br><i>Dilsea carnososa</i>                                                                                       |
| 4. <i>Codium</i>       | spring peak of production              | <i>Codium fragile</i><br><i>Halidrys siliquosa</i>                                                                                         |

The exact reasons for the different periods of peak antibiotic production are unknown, however some correlations exist. Uniform antibiotic production throughout the year could indicate that these compounds are merely secondary metabolites from some essential, major metabolic pathway. The winter peak of activity coincides with the period of minimum photosynthesis and general metabolic inactivity of the plant. At this stage it may be necessary for the plant to prevent the establishment of other organisms on their surfaces during this dormant period.

The summer peak of activity coincides with increased water temperatures and light intensity. Herbivory also increases as do the number of spores, larvae etc. present in the water. At this stage it seems necessary for the plant to protect itself against these invading organisms. The spring peak of activity is associated with increased metabolic activity. This is the period when all dormant forms start growing and reproducing and hence the seaweeds needs protection.

The overall variations in production of antimicrobial substances make it difficult to correlate these phenomena with biochemical and/or physiological processes. It is necessary for the active principle(s) to be identified first, before any correlation can be made. These seasonal variations partly account for some of the apparently contradictory results obtained by other workers who have collected samples at only one time of the year.

Table 3.1.2 Contradictory results of antibiotic activity from various workers.

| Species                      | Activity                        | Reference                  |
|------------------------------|---------------------------------|----------------------------|
| <i>Ulva lactuca</i>          | min. activity spring and summer | Hornsey and Hide, 1976     |
| <i>Ulva linza</i>            | min. activity winter            | Pratt <i>et al.</i> , 1951 |
| <i>Ascophyllum nodosum</i>   | min. activity autumn and winter | Hornsey & Hide, 1976       |
| <i>Ascophyllum nodosum</i>   | min. activity winter            | Vacca and Walsh, 1954      |
| <i>Laurencia pinnatifida</i> | activity throughout the year    | Hornsey & Hide, 1976       |
| <i>Laurencia obtusa</i>      | max. activity winter and spring | Almodovar, 1964            |

### 3.2 Materials and Methods:

The methods used in this screening programme were similar to those described in Section 2.2, with a few adaptations. Firstly methanol was used to extract the antimicrobial compounds, secondly, the paper disc technique was used to test activity and thirdly, a specific volume of extract was placed on each disc, this being 100µl.

The microorganisms used to test for activity were the same two Gram negative bacteria, three Gram positive and one yeast that are listed in Table 2.2.3.

A large seaweed collection was used which included representatives from each of the three major algal groups. These are listed in Table 3.2.1.

The seaweeds were collected from the First Sister, Riet River on the 16 April 1987, 11 August 1987 and 24 October 1987.

Table 3.2.1 The seaweeds used in the seasonal antimicrobial activity study.

| Group         | Genus & species                                                                                                                                                                                                                                                                                                                                                     |
|---------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Rhodophyceae  | <i>Galaxaura diesingiana</i> Zanardini<br><i>Gelidium amansii</i> (Lamouroux) Lamouroux<br><i>Gelidium pristoides</i> (Turner) Kuetzing<br><i>Peyssonelia capensis</i> Montagne<br><i>Gracilaria beckeri</i> (J. Agardh) Papenfuss<br><i>Plocamium corallorhiza</i> (Turner) Harvey<br><i>Hypnea spicifera</i> (Suhr) Harvey<br><i>Laurencia glomerata</i> Kuetzing |
| Phaeophyceae  | <i>Dictyopteris macrocarpa</i> (Areschoug) Schmidt<br><i>Dictyota naevosa</i> (Suhr) J. Agardh<br><i>Zonaria subarticulata</i> (Lamouroux) Papenfuss<br><i>Endarachne binghamiae</i> J. Agardh<br><i>Iyengaria stellata</i> (Boergesen) Boergesen<br><i>Ecklonia biruncinata</i> (Bory) Papenfuss<br><i>Sargassum heterophyllum</i> (Turner) C. Agardh              |
| Chlorophyceae | <i>Ulva rigida</i> C. Agardh<br><i>Halimeda cuneata</i> Hering                                                                                                                                                                                                                                                                                                      |

Due to the fact that many seaweeds grow seasonally and are not present all year round, some species could not be used for this study.

### 3.3 Results and Discussion:

Table 3.3.1 represents the antimicrobial activities of methanolic extracts of selected eastern Cape seaweeds which were collected at different times of the year. It should be noted, that the aim of this study was to do a full seasonal comparison of antimicrobial activities of the seaweeds listed in Table 3.2.1 from Autumn 1987 – Summer 1988. This however was not entirely possible, as unusually high tides and heavy seas were experienced close to the summer and winter solstices and the spring and autumn equinoxes. This made collections on the rocky outcrop of the First Sister at these specific times of the year impossible. As a result of this, only three collections were made during the yearly period these being on the 16 April 1987, 11 August 1987 and 24 October 1987.

Table 3.3.1.

Antimicrobial activity of methanolic extracts from selected eastern Cape intertidal seaweeds (Seaweeds collected at different times of the year as indicated).

Measurement = diameter of zone of inhibition in mm in excess of disc diameter

Legend: Sa = *Staphylococcus aureus*  
 Bs = *Bacillus subtilis*  
 Msp = *Micrococcus* sp.  
 Ec = *Escherichia coli*  
 Pa = *Pseudomonas aeruginosa*  
 Ca = *Candida albicans*

| MICROORGANISM<br>SEAWEED SPECIES | TIME OF YEAR |     |      |     |     |     |         |     |     |     |     |    |          |     |     |     |     |    |
|----------------------------------|--------------|-----|------|-----|-----|-----|---------|-----|-----|-----|-----|----|----------|-----|-----|-----|-----|----|
|                                  | 16/4/87      |     |      |     |     |     | 11/8/87 |     |     |     |     |    | 24/10/87 |     |     |     |     |    |
|                                  | Sa           | Bs  | Msp  | Ec  | Pa  | Ca  | Sa      | Bs  | Msp | Ec  | Pa  | Ca | Sa       | Bs  | Msp | Ec  | Pa  | Ca |
| RHODOPHYTA                       |              |     |      |     |     |     |         |     |     |     |     |    |          |     |     |     |     |    |
| <i>Galaxaura diesingiana</i>     | 0            | 3.6 | 0    | 5.5 | 0   | 0   | 0       | 0   | 1   | 0   | 0   | 0  | 3.7      | 0   | 1   | 0   | 0   | 0  |
| <i>Gelidum amansii</i>           | 0            | 0   | 0    | 6.1 | 0   | 0   | 5.9     | 0   | 0   | 0   | 0   | 0  | 4.7      | 0   | 2.3 | 0   | 0   | 0  |
| <i>Gelidium pristoides</i>       | 0            | 2.5 | 0    | 3   | 0   | 0   | 5.2     | 0   | 6   | 0   | 0   | 0  | 4.2      | 0   | 4   | 0   | 0   | 0  |
| <i>Peyssonnelia capensis</i>     | 3.3          | 3.8 | 0    | 0   | 0   | 2   | 3       | 0   | 0.3 | 0   | 0   | 0  | 0        | 0   | 2   | 0   | 0   | 0  |
| <i>Gracilaria beckeri</i>        | 1.7          | 0   | 3.1  | 0   | 0   | 0   | 4.3     | 0   | 1   | 0   | 0   | 0  | 5.2      | 0   | 2.8 | 0   | 0   | 0  |
| <i>Plocamium corallorhiza</i>    | 1            | 1.8 | 3.8  | 0   | 0   | 1   | 8.8     | 2.5 | 0   | 4.2 | 2.7 | 3  | 9.3      | 4.3 | 3.5 | 5.3 | 3.3 | 7  |
| <i>Hypnea spicifera</i>          | 0            | 0   | 0    | 3.7 | 0   | 0   | 0       | 0   | 0   | 0   | 0   | 0  | 3        | 0   | 1   | 0   | 0   | 0  |
| <i>Laurencia glomerata</i>       | 5.3          | 0   | 1    | 0   | 1   | 0   | 7       | 4.2 | 3.8 | 4.9 | 4.8 | 0  | 3.8      | 0   | 0.5 | 0   | 0   | 0  |
| PHAEOPHYTA                       |              |     |      |     |     |     |         |     |     |     |     |    |          |     |     |     |     |    |
| <i>Dictyopteris macrocarpa</i>   | 1.6          | 0   | 0    | 0   | 0   | 0   | 0       | 0   | 0   | 0   | 0   | 0  | 3.2      | 0   | 0   | 0   | 0   | 0  |
| <i>Dictyota naevosa</i>          | 4            | 0   | 6    | 0   | 0   | 0   | 4.6     | 0   | 3.3 | 0   | 0   | 0  | 0        | 0   | 8.3 | 0   | 0   | 0  |
| <i>Zonaria subarticulata</i>     | 7.4          | 0   | 10.1 | 0   | 0   | 0   | 12.3    | 0   | 14  | 0   | 4.6 | 0  | 11.5     | 0   | 8.5 | 0   | 4   | 0  |
| <i>Endarachne binghamiae</i>     | 0            | 0   | 0.7  | 0   | 0   | 0   | 0       | 0   | 1.3 | 0   | 0   | 0  | 0        | 0   | 2   | 0   | 0   | 0  |
| <i>Iyengaria stellata</i>        | 0            | 0   | 0    | 0   | 0   | 0   | 0       | 0   | 0   | 0   | 0   | 0  | 0        | 0   | 0   | 0   | 0   | 0  |
| <i>Ecklonia biruncinata</i>      | 11           | 3   | 2.5  | 4   | 2.3 | 0   | 9       | 1   | 1.5 | 0   | 1.8 | 0  | 11       | 0   | 3.3 | 4.1 | 3.8 | 0  |
| <i>Sargassum heterophyllum</i>   | 4.3          | 0   | 4.2  | 0   | 0   | 0   | 4.2     | 0   | 4.2 | 0   | 0   | 0  | 0        | 0   | 1.9 | 0   | 0   | 0  |
| CHLOROPHYTA                      |              |     |      |     |     |     |         |     |     |     |     |    |          |     |     |     |     |    |
| <i>Ulva rigida</i>               | 6.3          | 0   | 1    | 0   | 0   | 2.8 | 0       | 0   | 0   | 0   | 0   | 0  | 4        | 0   | 2.5 | 0   | 0   | 0  |
| <i>Halimeda cuneata</i>          | 0            | 0   | 2.2  | 0   | 0   | 0   | 0       | 0   | 2.7 | 0   | 0   | 0  | 1        | 0   | 1.6 | 0   | 0   | 0  |

It is difficult to assess the data shown in Table 3.3.1 at a glance, hence further analysis has been carried out.

Three variables were analysed for their effect on the observed antimicrobial activities of seaweed extracts, these were:

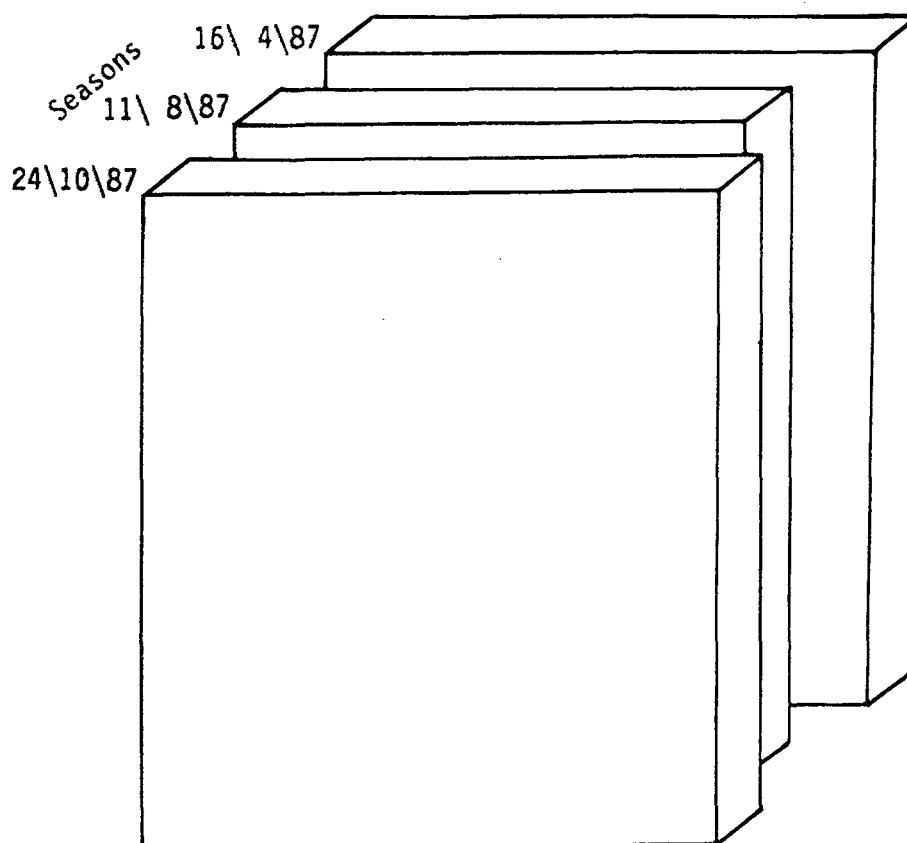
- (i) Season
- (ii) Seaweed species
- (iii) Microorganism species

To assess the seasonal data, the seaweed and microorganism data were pooled. Table 3.3.2 draws a comparison between the antimicrobial activity of the seaweed extracts at the different collection periods, whereas Fig. 6 shows this data graphically.

Table 3.3.2. A comparison of seasonal antimicrobial data

| Time of Year | Inhibition events<br>(possible 102) | Seasonal potency<br>index (SPI) |
|--------------|-------------------------------------|---------------------------------|
| 16/4/87      | 35                                  | 45                              |
| 11/8/87      | 31                                  | 40                              |
| 24/10/87     | 34                                  | 43                              |

Potency index 1 = (zone of inhibition 7-10mm)  
 2 = (zone of inhibition 11-15mm)  
 3 = (zone of inhibition 16-20mm)



Seasonal Potency of Extract Inhibition Index in mm.

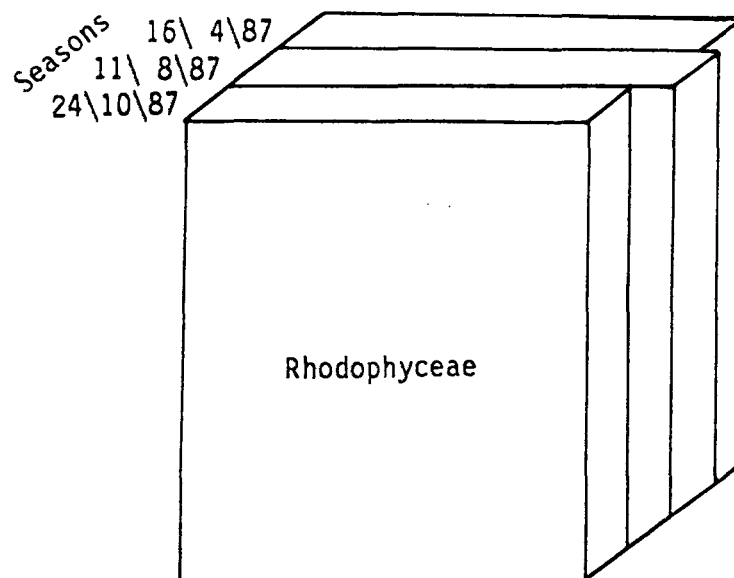
Fig. 6 A graphic representation of the seasonal antimicrobial data (Table 3.3.2).

From Fig. 6, the antimicrobial activity appears to be marginally less in winter than in spring and autumn. This result however is not significant, and so at  $P=0.9$ , it is possible to conclude that no significant difference in antimicrobial activity exists between the seaweeds collected at the three different times of the year i.e. 16/4/87, 11/8/87 and 24/10/87.

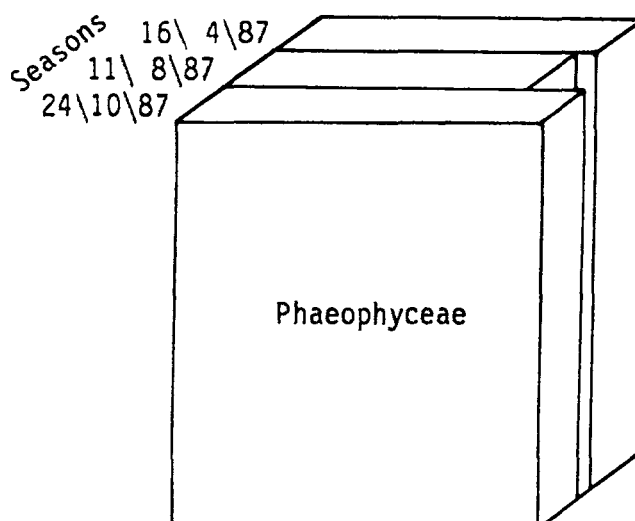
To assess the seasonal data of each of the three major algal groups, Table 3.3.3 was drawn up.

Table 3.3.3. A comparison of seasonal antimicrobial data for the three major algal groups.

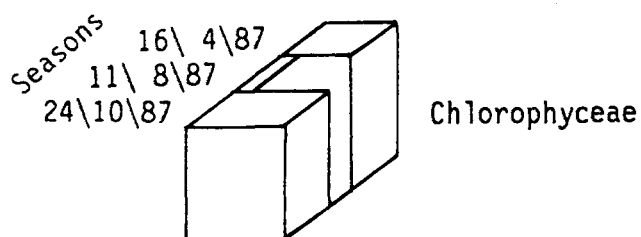
|             | Time of Year | Inhibition events | Seasonal potency index (SPI) |
|-------------|--------------|-------------------|------------------------------|
| Rhodophyta  | 16/4/87      | 18                | 21                           |
|             | 11/8/87      | 18                | 23                           |
|             | 24/10/87     | 19                | 22                           |
| Phaeophyta  | 16/4/87      | 13                | 19                           |
|             | 11/8/87      | 12                | 16                           |
|             | 24/10/87     | 11                | 17                           |
| Chlorophyta | 16/4/87      | 4                 | 5                            |
|             | 11/8/87      | 1                 | 1                            |
|             | 24/10/87     | 4                 | 4                            |



Seasonal Potency of Extract Inhibition in mm



Seasonal Potency of Extract Inhibition in mm



Seasonal Potency of Extract Inhibition in mm

Fig. 7 A graphic representation of the seasonal antimicrobial data (Table 3.3.3) for the three major algal groups.

The Rhodophyceae show a slight increase in antimicrobial activity in winter, whereas both the Phaeophyceae and Chlorophyceae show reduced activity in winter. These differences are very slight, and therefore would not be considered significant, particularly that of the Chlorophyceae which has a very small sample size.

In a comparison done on the antimicrobial activities of the different seaweed species (2nd variable) the seasonal and microorganism data were pooled. Tables 3.3.4 – 3.3.6 were drawn up, and Figs. 8-10 were derived from these tables.

Table 3.3.4 The antimicrobial activity of the Rhodophycean species.

| RHODOPHYTA                       | Inhibition events<br>(possible 18) | Extract potency<br>index |
|----------------------------------|------------------------------------|--------------------------|
| 1. <i>Galaxaura diesingiana</i>  | 5                                  | 6                        |
| 2. <i>Gelidium amansii</i>       | 4                                  | 7                        |
| 3. <i>Gelidium pristoides</i>    | 6                                  | 8                        |
| 4. <i>Peyssonnelia capensis</i>  | 6                                  | 6                        |
| 5. <i>Gracilaria beckeri</i>     | 6                                  | 7                        |
| 6. <i>Plocamium corallorhiza</i> | 15                                 | 19                       |
| 7. <i>Hypnea spicifera</i>       | 3                                  | 3                        |
| 8. <i>Laurencia glomerata</i>    | 10                                 | 12                       |

Potency index 1 = (zone of inhibition 7-10mm)  
 2 = (zone of inhibition 11-15mm)  
 3 = (zone of inhibition 16-20mm)

Table 3.3.5 The antimicrobial activity of the Chlorophycean species

| PHAEOPHYTA                         | Inhibition events<br>(possible 18) | Extract potency<br>index |
|------------------------------------|------------------------------------|--------------------------|
| 9. <i>Dictyopteris macrocarpa</i>  | 2                                  | 2                        |
| 10. <i>Dictyota naevosa</i>        | 5                                  | 7                        |
| 11. <i>Zonaria subarticulata</i>   | 8                                  | 18                       |
| 12. <i>Endarachne binghamiae</i>   | 3                                  | 3                        |
| 13. <i>Iyengaria stellata</i>      | 0                                  | 0                        |
| 14. <i>Ecklonia biruncianata</i>   | 13                                 | 18                       |
| 15. <i>Sargassum heterophyllum</i> | 5                                  | 5                        |

Potency index 1 = (zone of inhibition 7-10mm)  
 2 = (zone of inhibition 11-15mm)  
 3 = (zone of inhibition 16-20mm)

Table 3.3.6 The antimicrobial activity of the Chlorophycean species

| CHLOROPHYTA                 | Inhibition events<br>(possible 18) | Extract potency<br>index |
|-----------------------------|------------------------------------|--------------------------|
| 16. <i>Ulva rigida</i>      | 5                                  | 6                        |
| 17. <i>Halimeda cuneata</i> | 4                                  | 4                        |

Potency index 1 = (zone of inhibition 7-10mm)  
 2 = (zone of inhibition 11-15mm)  
 3 = (zone of inhibition 16-20mm)

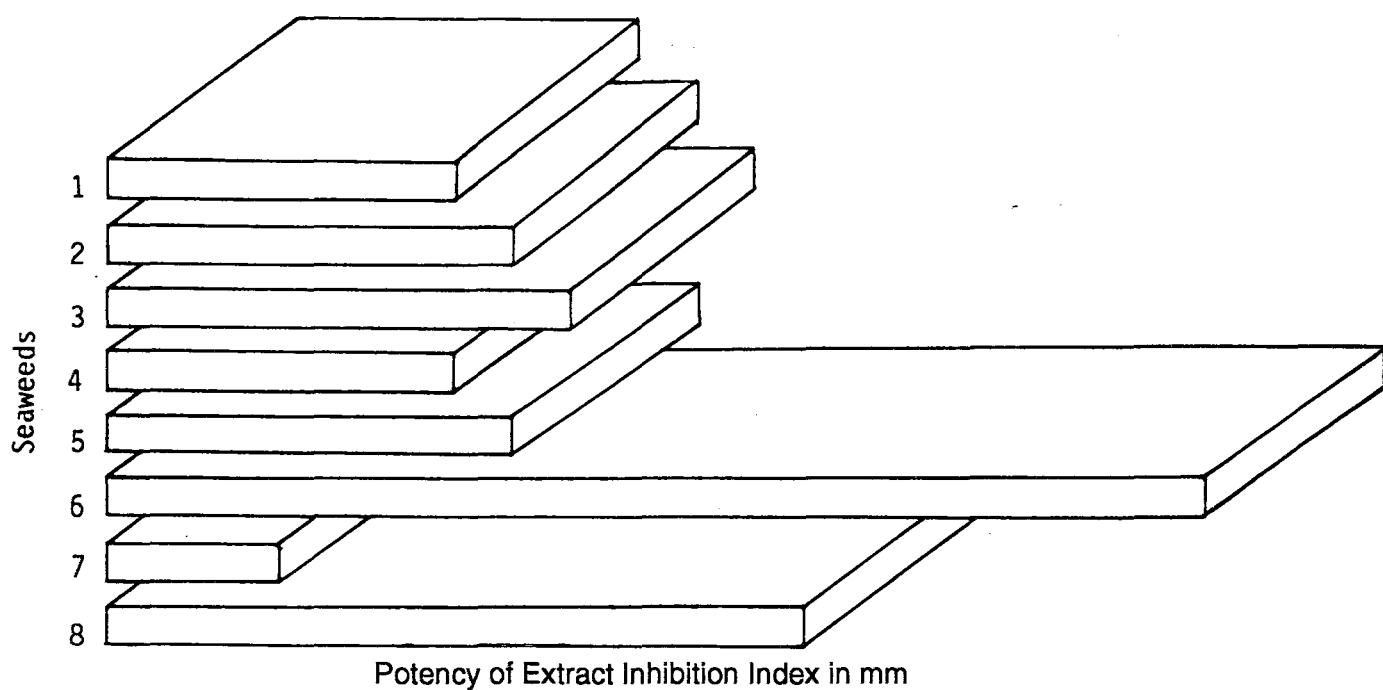


Fig.8 A graphic representation of the antimicrobial activity (Table 3.3.4) of the Rhodophycean species

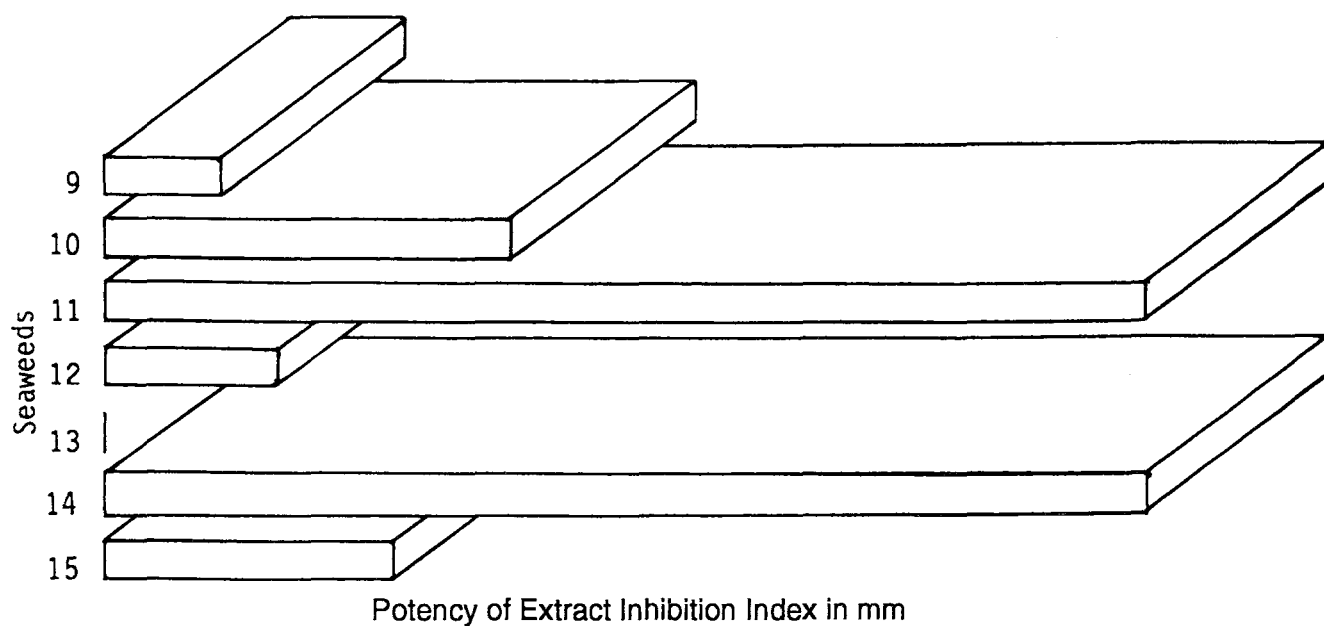


Fig. 9 A graphic representation of the antimicrobial activity (Table 3.3.5) of the Phaeophycean species.

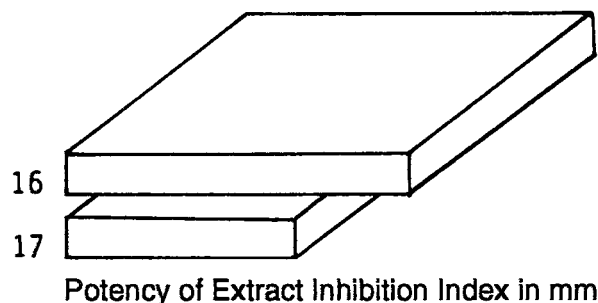


Fig 10. A graphic representation of the antimicrobial activity (Table 3.3.6) of the Chlorophycean species.

The extract potency index (EPI) shows that in the case of *H.spicifera* for example (Table 3.3.4), all three positive inhibition events recorded in the first column were between 7-10mm diameter i.e. weak potency. *P.corallorhiza* however shows more potent activity within the 15 positive inhibition events i.e. EPI = 19.

Fig. 8 shows very high bioactivity from the Rhodophyte *P.corallorhiza*, with *Z.subarticulata* and *E.biruncinata* both Phaeophytes showing equally high activity. (Fig. 9). To assess whether significant differences ( $P < 0.001$ ) in activity occurred between the different seaweed extracts, a table of the most potent extracts was drawn up and a bioactive index used to compare activities.

Table 3.3.7 A comparison of the most potent antimicrobially active seaweed extracts.

| Seaweeds                      | Bioactivity index |
|-------------------------------|-------------------|
| <i>Zonaria subarticulata</i>  | 1.0 (14mm)        |
| <i>Ecklonia biruncinata</i>   | 0.8               |
| <i>Plocamium corallorhiza</i> | 0.7               |
| <i>Dictyota naevosa</i>       | 0.6               |
| <i>Laurencia glomerata</i>    | 0.5               |
| <i>Rest</i>                   | <0.4              |

To determine the bioactive index (BI), the largest diameter of inhibition recorded (14mm) was arbitrarily assigned the value 1, and all subsequent values were derived from that.

The above table confirms the results obtained from Figs. 8-10.

It was necessary, to determine whether any particular orders of the seaweeds used showed particularly high activity compared with the others. The most bioactive orders were assessed, producing Table 3.3.8.

Table 3.3.8 The bioactivity of the various orders of seaweeds tested.

| Order (no. of species) | Bioactive index |
|------------------------|-----------------|
| Dictyotales (3)        | 1.0 (14mm)      |
| Laminariales (1)       | 0.8             |
| Gigartinales (3)       | 0.7             |
| Ceramiales (1)         | 0.5             |
| Rest                   | < 0.45          |

Some orders did show significantly greater bioactivity than others ( $P < 0.001$ ). The most bioactive order was:

Dictyotales: *Zonaria subarticulata*  
*Dictyota naevosa*  
*Dictyopteris macrocarpa*

followed by

Laminariales: *Ecklonia biruncinata*

Both these orders are members of the Phaeophyceae, which are known to produce phenols, which are toxic to organisms, as they bind and inactivate proteins.

The other active orders are members of the Rhodophyceae:

Gigartinales: *Gracilaria beckeri*  
*Plocamium corallorhiza*  
*Hypnea spicifera*

Ceramiales: *Laurencia glomerata*

Studies carried out by Caccamese and Azzolina (1979) showed antimicrobial activity to be concentrated in the Dictyotales and Fucales of the Phaeophyceae, and the Ceramiales of the Rhodophyceae, with the Cryptonemiales and Gigartinales, both Rhodophyceae, showing smaller amounts of activity.

As shown in Table 3.3.8, the Dictyotales have the strongest activity in the South African Phaeophyceae species tested as well. The Fucales (*S.heterophyllum*) however did not show strong activity. Of the Rhodophyceae, the Gigartinales showed stronger activity than the Ceramiales, however the small sample size should be taken into account.

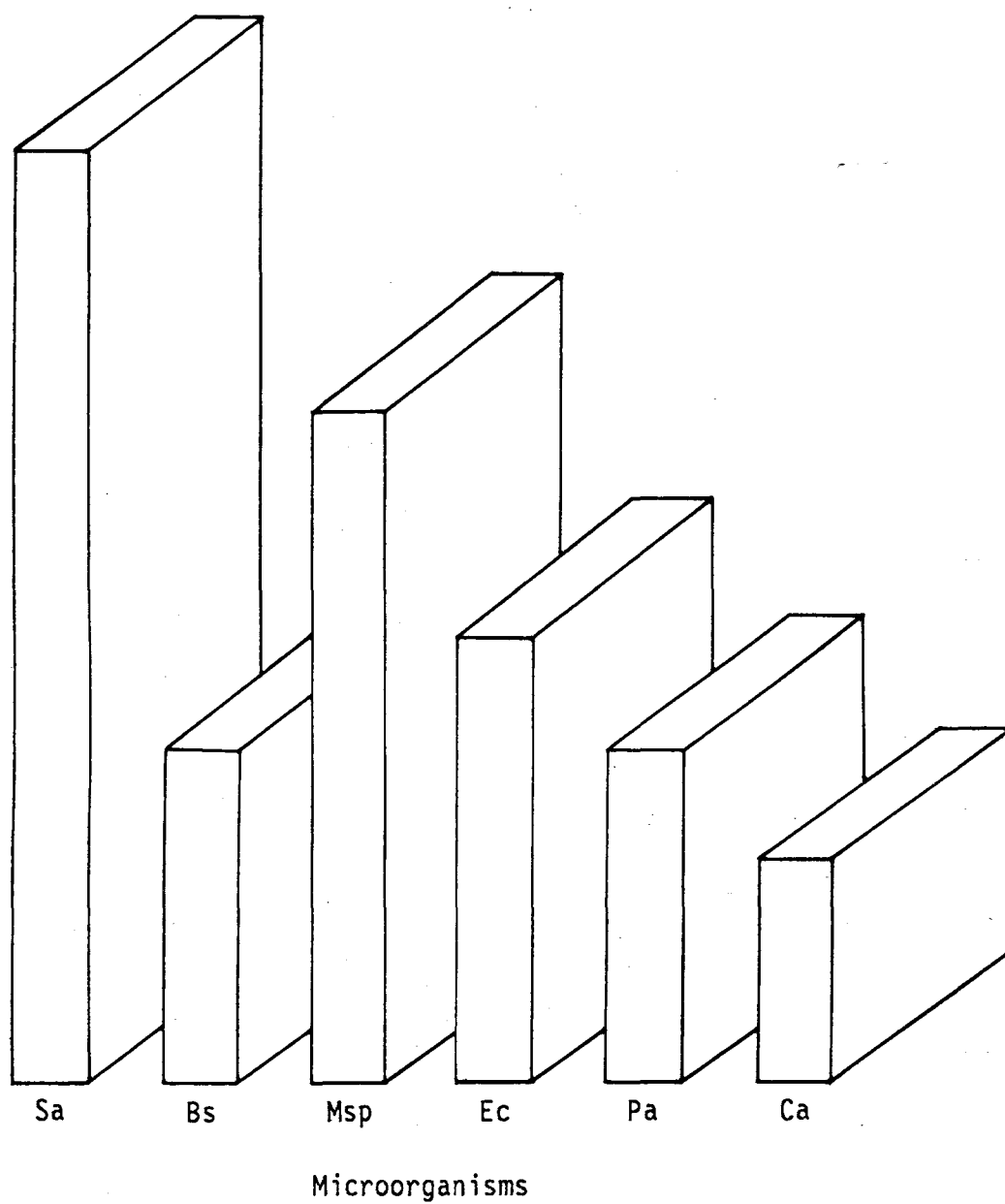
The third variable was the microorganism species. A comparison of the varying susceptibilities of the microorganisms used, to the seaweed extracts, is represented in Table 3.3.9.

Table 3.3.9 The susceptibility of test microorganisms to seaweed extracts.

| Gram Positive Bacteria        | Inhibition events<br>(possible 51) | Strain susceptibility<br>index |
|-------------------------------|------------------------------------|--------------------------------|
| <i>Staphylococcus aureus</i>  | 32                                 | 50                             |
| <i>Bacillus subtilis</i>      | 9                                  | 9                              |
| <i>Micrococcus species</i>    | 36                                 | 43                             |
| Gram Negative Bacteria        |                                    |                                |
| <i>Escherichia coli</i>       | 9                                  | 12                             |
| <i>Pseudomonas aeruginosa</i> | 9                                  | 9                              |
| Yeast                         |                                    |                                |
| <i>Candida albicans</i>       | 5                                  | 6                              |

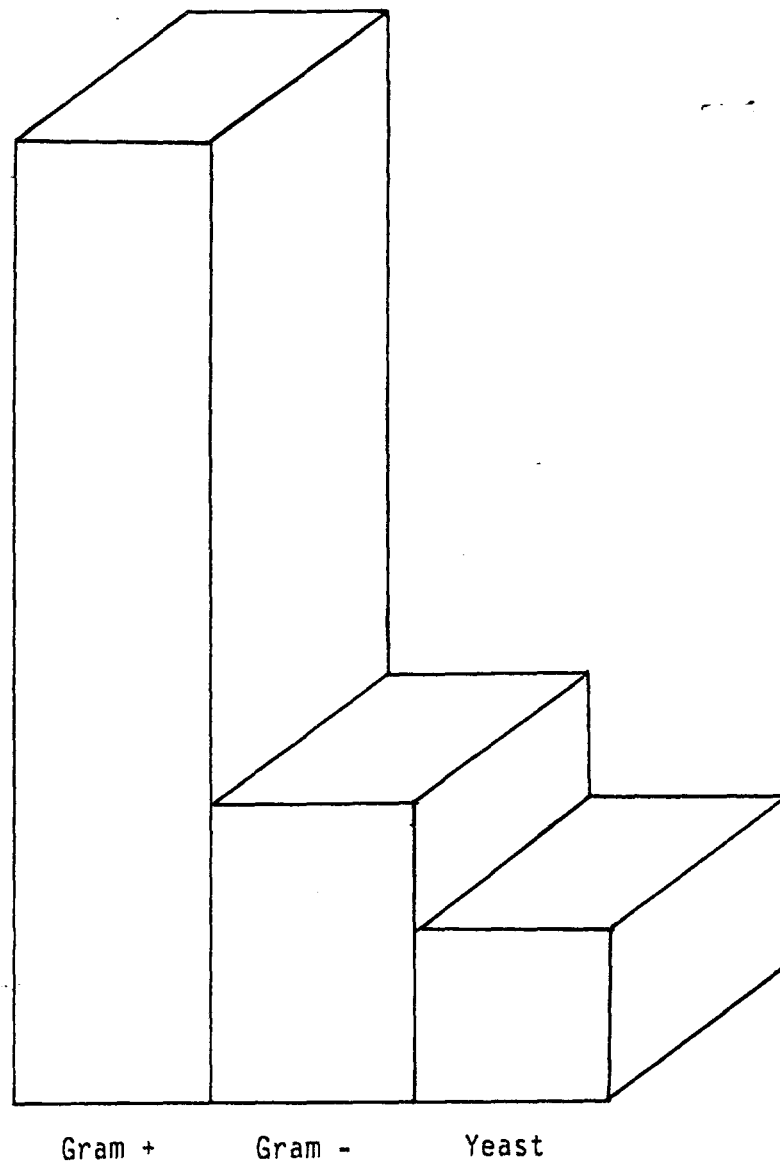
Susceptibility index    1 = (zone of inhibition 7-10mm)  
                                   2 = (zone of inhibition 11-15mm)  
                                   3 = (zone of inhibition 16-20mm)

The strain susceptibility index (SSI) has been derived in the same way as the EPI. In this case, the susceptibility of the microorganism strain to the seaweed extract is being determined. The data from Table 3.3.9 is represented in Figs. 11 and 12.



Potency of Strain Susceptibility Index in mm

Fig. 11 A graphic representation of the susceptibility of the test microorganisms to seaweed extracts



Potency of Strain Susceptibility Index in mm

Fig. 12 A graphic representation of the susceptibility of the test microorganisms, as groups, to seaweed extracts.

Fig. 11 clearly indicates that the Gram positive *S.aureus* was the most susceptible organism to treatment with seaweed extracts. The *Micrococcus* species was also highly susceptible to treatment, with the high resistance shown by the Gram positive *B.subtilis* probably being due to *B.subtilis* being an endospore former. The production of endospores occurs as the culture passes out of the exponential growth phase, and this is accompanied by increased thermostability, and general resistance to treatment. There is therefore a significant difference in susceptibility between species of microorganisms ( $P<0.001$ ). See Table 3.3.10.

Table 3.3.10 A comparison of the susceptibilities of microorganism species to seaweed extracts.

| Microorganism                | Susceptibility index |
|------------------------------|----------------------|
| <i>Staphylococcus aureus</i> | 1.0 (12.3mm)         |
| <i>Micrococcus</i> species   | 0.8                  |
| rest                         | <0.5                 |

The organisms were grouped into yeast, Gram positive and Gram negative bacteria to determine whether these groups of organisms showed any general trends in susceptibility (Fig. 12). It appears therefore, that the two Gram positive bacteria (*S.aureus* and *Micrococcus* sp.) were more susceptible to treatment, whereas the Gram negatives (*E.coli* and *P.aeruginosa*) were more resistant. The yeast *C.albicans* was resistant to all extracts except for *P.capensis*, *P.corallorhiza* and *U.rigida*. (Table 3.3.1)

As a result of the above studies, it is apparent, that no clear seasonal changes in antimicrobial activity occurred in the seaweeds which were screened. Of the seaweeds tested, some however are worth mentioning for their potent broad spectrum antimicrobial activity, these are the two Rhodophyceae, *Plocamium corallorhiza* and *Laurencia glomerata*, and the two Phaeophyceae *Zonaria subarticulata* and *Ecklonia biruncinata*. Of these, *L.glomerata* and *E.biruncinata* were effective against all the test organisms except *C.albicans*.

## CHAPTER IV

### A CORRELATION OF SCANNING ELECTRON MICROSCOPIC INFORMATION AND ANTIBIOSIS.

#### 4.1 Introduction:

In the past, researchers of natural products have been driven by the need to discover new compounds which could be of benefit to man. Terrestrial plants have a long history of medical application, unlike the marine organisms. Even so, marine organisms have left a record of hazardous effects to mankind. Saxitoxin, tetrodotoxin and lyngbyatoxin are three examples of marine products which have caused problems. One aspect of marine research which has been neglected, are the benefits that these secondary products, and others have for the producing organism. Secondary metabolites may serve a number of functions – they may discourage herbivory by molluscs, crustaceans and fish and prevent algal-algal, -seagrass, -epiphyte, -epizoic animal, -bacterial and -fungal interactions [Littler and Littler, 1985]. The above are referred to as fouling agents as they reduce growth and survival of the marine organisms. They also influence interspecific competition due to space reduction, and increased drag on the algae, with the result that community structure is affected [Lobban *et al.*, 1985].

In more recent years interest has developed in the chemical ecology of marine organisms. The early discovery of algal sex attractants resulted in many research groups entering this field of study. Studies of bioactive metabolites from terrestrial plants, has resulted in the development of theories on plant-herbivore interactions and biochemical evolution. These studies suggest that the development of algal defense mechanisms are related to a number of factors;

- (i) the risk the plant runs of being discovered by herbivores
- (ii) the cost of defense to the plant on an energy utilization scale
- (iii) the importance of various parts of the plant to its survival.

The role of secondary metabolites as chemical defense compounds is generally accepted. However there is still considerable speculation as to how the physical environment and predatory herbivores interact to affect plant chemistry. These biochemical interactions and intricate mechanisms which exist between the algae and the environment are obviously highly complex, yet very subtle.

#### 4.2 Materials and Methods:

##### 4.2 (a) Scanning Electron Microscopy:

Scanning Electron Microscopy (SEM) can be used to study seaweeds, to determine to what extent their surfaces are contaminated/fouled by microorganisms etc. One of the fundamental problems of SEM is that the specimen chamber demands that the specimen be dry, if not frozen. If they are not already dry, the specimens are soon subjected to rapid vacuum desiccation in the specimen chamber which is likely to result in distortion of all but the toughest tissue [Cross, 1987]. Natural drying (in air) or drying aided by dehydrating agents results in distortion which arises from the surface tension forces applied to cell walls etc. as the liquid/vapour phase interface passes through the specimen. Soft hydrated biological specimens allowed to dry in this way are characterised by massive cell collapse and shrinkage due to

these forces. Critical point drying prevents this from happening by preventing the liquid/vapour interface from passing through the specimen. This is accomplished by raising the temperature and pressure of the liquid in and surrounding the specimen to above its critical point. By doing this, there is no longer an interface between the liquid and vapour phases. The temperature is then maintained above the critical point and the pressure is lowered to atmospheric pressure. This results in the liquid which previously filled and surrounded the specimen being bled off as a vapour. When the vessel is opened, a perfectly dry specimen with minimal distortion remains. The critical temperature and pressure of water and most dehydrating agents is very high. It is therefore necessary to use other solvents with lower critical points, which do not damage the specimens. The alternative, is the manufacture of suitable equipment to withstand very high temperatures and pressures. This would be impracticable therefore other solvents are used. Liquid carbon dioxide is most commonly used. It has a critical point temperature of 31°C and a pressure of 73.8 atmospheres. It is also non-toxic, inexpensive and fairly inert. One major disadvantage of liquid CO<sub>2</sub> however, is that it is not miscible with ethanol. It is therefore necessary to introduce a transitional solvent, namely amyl acetate, which is miscible with both liquid CO<sub>2</sub> and ethanol. Although critical point drying gives good structural preservation of all tissue types, including the softest most delicate tissues, some shrinkage does occur, making measurements taken from critical point dried specimens inaccurate.

Once critical point dried, it is necessary to coat the surface of the specimens with a thin layer of metal or metal alloy e.g. gold or gold palladium. This improves the secondary electron emission from biological materials in the SEM, and reduces the charge by increasing the electrical conductivity of the specimen surface.

Various methods are used for metal coating, in this case sputter coating was used. The principle of sputter-coating is that if an inert gas e.g. argon is placed in a partial vacuum and an electric field is applied, the residual molecules become ionized resulting in rapid movement. These molecules can bombard a target, namely a sheet of gold foil, to such an extent, that gold atoms are released. The presence of the electric field and the ionized gas molecules result in the gold atoms moving in the direction away from the gold target, towards the opposite pole, where the specimen has been placed. This results in the specimen being coated with gold. The advantages of this method of metal coating are its speed, its relatively low cost, and the uniformity of coating that results. The disadvantages are that the specimen is heated, and at high magnification, the coating results in a granular appearance [Cross, 1987].

#### 4.2 (b) Critical Point Drying Procedure:

The pieces of seaweed (1mm<sup>3</sup>) were placed in cold buffered fixative (gluteraldehyde) in labelled vials and allowed to fix for a minimum of 12 hours. The fixative was decanted off and replaced with cold phosphate buffer twice and allowed to wash for 10 minutes. All the solutions used were maintained at 4°C until after the 80% ethanol stage. The phosphate buffer was decanted off, and replaced with 30% ethanol, then allowed to dehydrate for 5 minutes. This procedure was repeated for each of the ethanol series, 50%, 70%, 80%, 90% and twice for 100% ethanol. After the 100% ethanol was decanted off, a 75:25 ethanol:amyl acetate mixture was added and left for 20 minutes. This procedure was repeated for the 50:50 and 25:75 ethanol:amyl acetate solutions after which pure amyl acetate was added. The

specimens were then transferred to the critical point drying (CPD) baskets after 20 minutes, and placed in fresh 100% amyl acetate. The CPD equipment used was Polaron E 3000 series II. The CPD procedure involved the bleeding off of amyl acetate and the introduction of liquid CO<sub>2</sub> which was followed by an increase in temperature and pressure. This resulted in the disappearance of the liquid phase, which took approximately 2 hours to complete. This was followed by the CO<sub>2</sub> being bled off at high temperature, to prevent the CO<sub>2</sub> from condensing in the specimen. The specimens were then removed, and stored in a desiccator ready for mounting on stubs, and metal coating [Cross, 1987].

#### 4.2 (c) Metal coating:

Sputter coating involved placing the specimens in the sputter coater holder, which was clamped in position, and the chamber closed. The sputter coating equipment used was a Polaron SEM Coating Unit E 5100. The chamber was evacuated until a vacuum better than  $5 \times 10^{-2}$  Torr was reached. The sputter current was allowed to rise to approximately 15mA for 90 seconds and sputtering proceeded. After the coating was complete, air was admitted into the chamber. Care was taken at this stage to prevent water vapour condensing on the specimens. Once removed from the holder, the specimens were ready for examination in the SEM.

The specimens were mounted onto stubs using chloroform-based adhesive which was allowed to dry. The seaweeds were then viewed, and the presence of microorganisms on the seaweed surfaces were recorded on micrographs.

#### 4.2 (d) Microorganism assay:

Nutrient and Wickerham's agar plates were prepared as in section 2.2. When the agar had solidified, whole pieces of seaweed were placed on the agar surface. These were incubated at 37°C for 24-48 hrs so that any microorganisms present on the surfaces of the seaweeds could grow. The microorganisms that resulted were subcultured, and then identified firstly by staining techniques and then confirmation of these results were obtained from the South African Medical Institute for Research (SAMIR), Grahamstown.

#### 4.2 (e) Gram Stain Procedure:

The preliminary differentiation of bacteria was made on the basis of their reaction to staining procedures. The Gram stain differentiates nearly all bacteria into two major subgroups of procaryotes that differ with respect to the nature of the cell wall.

The Gram staining procedure was carried out by preparing a bacterial smear by placing a small amount of culture onto a glass slide, then allowing it to dry. By passing the smear through a flame several times it becomes fixed to the glass. The procedure then involved covering the smear with crystal violet reagent. After one minute, the slide was rinsed in slow running tap water. This was followed by rinsing with iodine reagent, the excess of which was poured off, then the smear was covered in iodine and allowed to stand for one minute. The slide was rinsed again, then decolourized with acetone. This reagent was added until the dye ran clear. This rinsing was followed by staining with safranin for 30 seconds. After rinsing again as above, the slide was blotted dry and was then ready for observation under the microscope [Stanier *et al.*, 1980].

Due to the nature of the bacterial cell walls, the Gram positive bacteria take up the blue stain and as a result are not decolourized, whereas the Gram negative bacteria do not hold the blue stain, so are counterstained with safranin which leaves a red colour.

#### 4.3 Results and Discussion:

Micrographs were taken of various parts of the algae, namely tip, middle and base of the plant. There appeared to be no variation in the concentration of bacteria on the surfaces of these algae. The following micrographs show the tip and base views of three of the brown seaweeds, *Zonaria subarticulata*, *Ecklonia biruncinata* and *Sargassum heterophyllum*.



Fig. 13 The micrograph showing the tip view of *Zonaria subarticulata*.

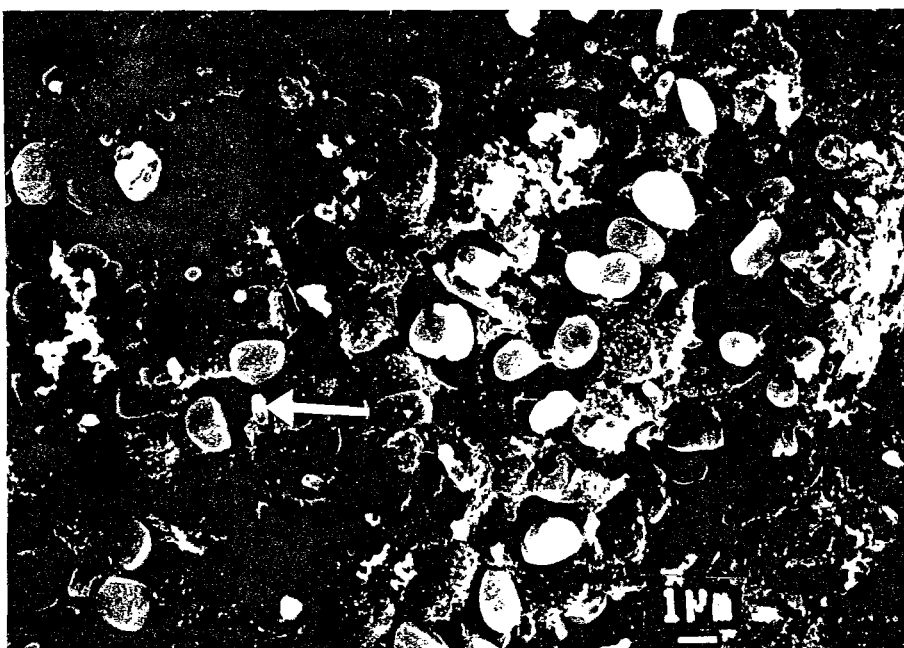


Fig. 14 The micrograph showing the base view of *Zonaria subarticulata*.

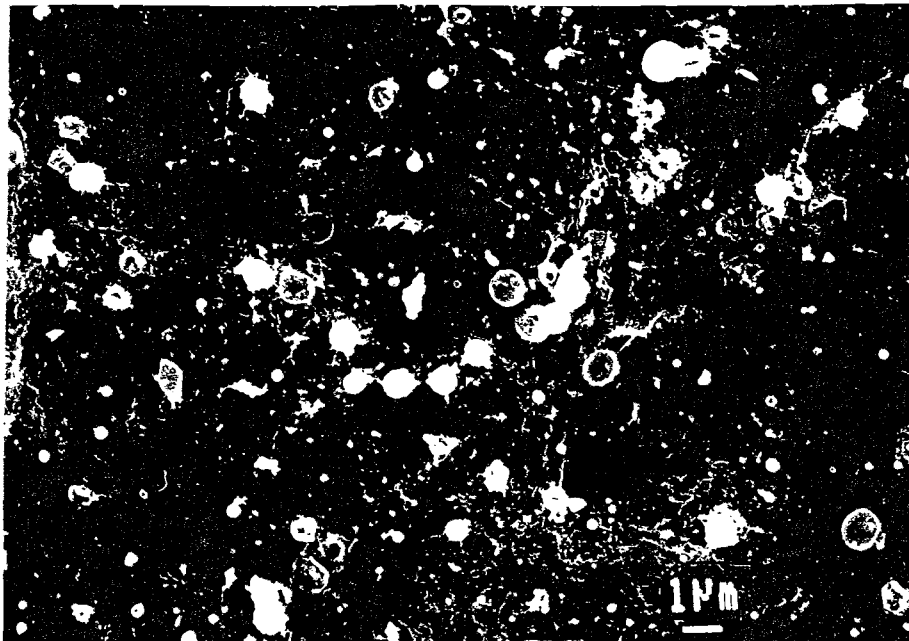


Fig. 15 The micrograph showing the tip view of *Ecklonia biruncinata*.

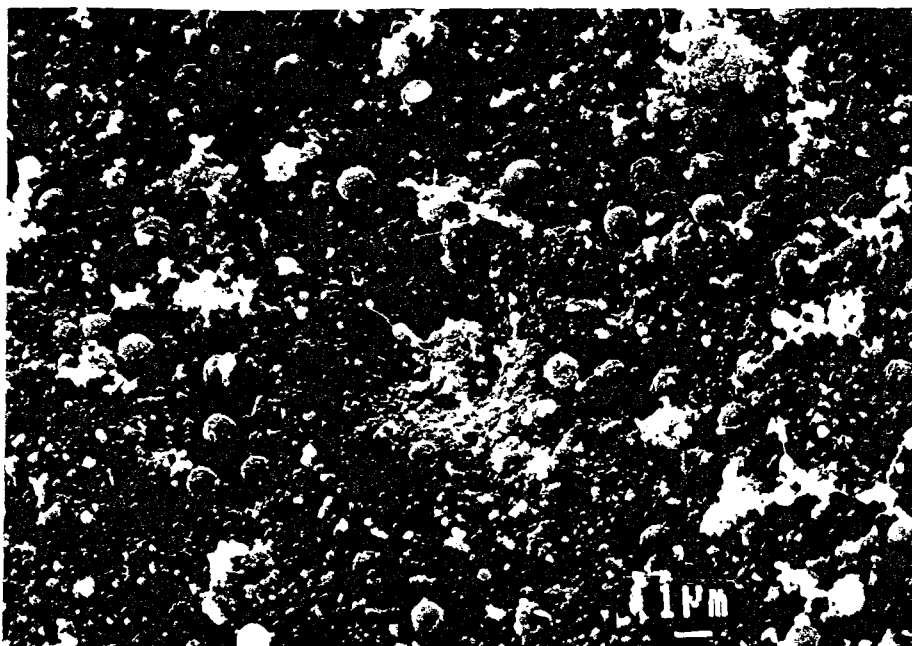


Fig. 16 The micrograph showing the base view of *Ecklonia biruncinata*.

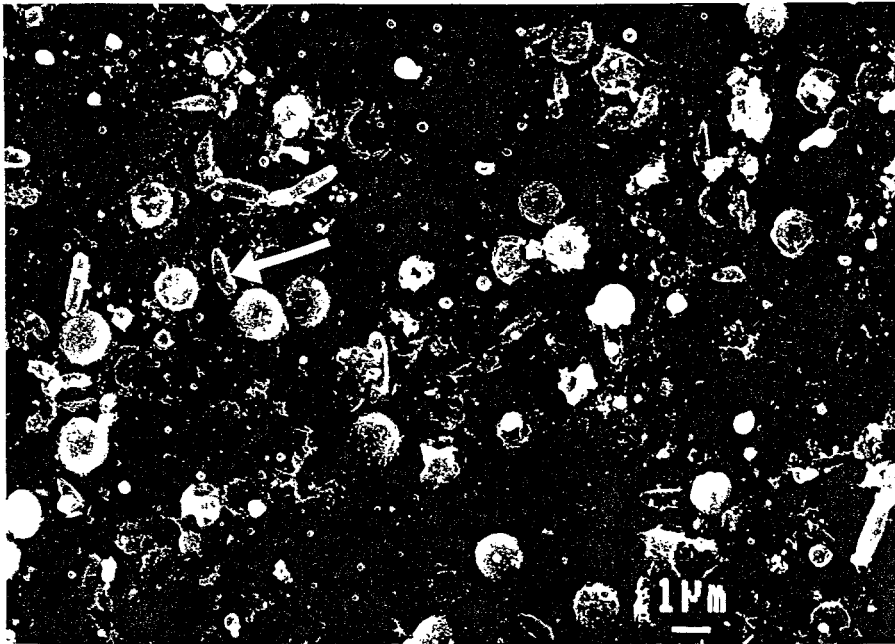


Fig. 17 The micrograph showing the tip view of *Sargassum heterophyllum*.

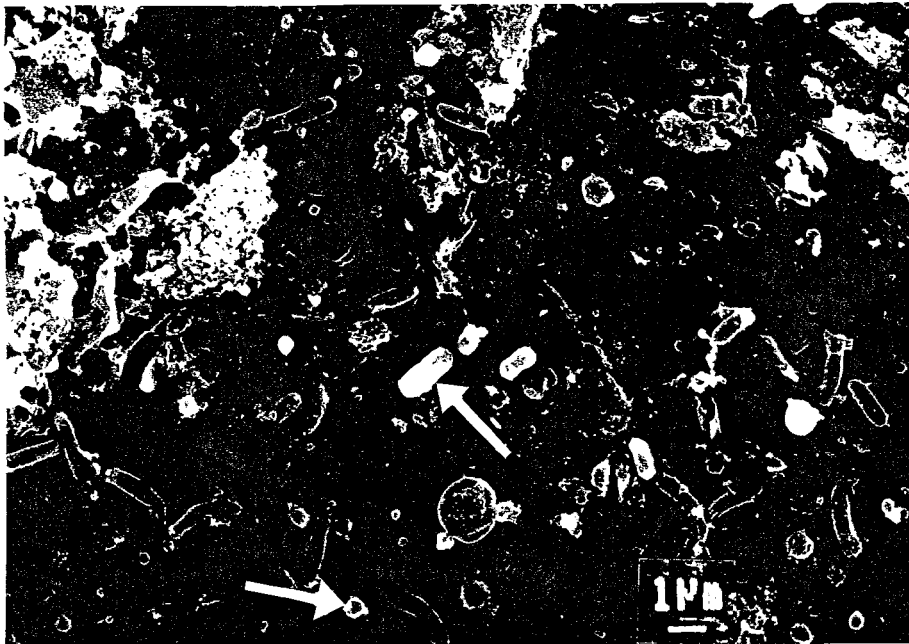


Fig. 18 The micrograph showing the base view of *Sargassum heterophyllum*.

In the microorganism study in Section 4.2 (d), only the organism *B.subtilis* was found to be present on the algal surfaces. These organisms are visible on the micrographs as short rod shaped structures; these however may be photographed end-on and hence will appear circular in shape. The identification of this organism was confirmed by the South African Medical Institute for Research (SAMIR), Grahamstown.

The results shown in Table 3.3.1 confirm that the *Z.subarticulata* and *S.heterophyllum* extracts were inactive against *B.subtilis*, with the *E.biruncinata* extract being slightly active in autumn and winter, with no activity being present in spring. As the seaweeds used for the micrographs were collected in spring, these seaweeds would be inactive against *B.subtilis*, and hence the presence of rod-shaped organisms would be expected. The *E.biruncinata* micrographs however, appear almost devoid of rod-shaped structures, but have a large number of circular or coccal-shaped organisms on their surfaces. The *S.heterophyllum* tip micrograph also appears to be harbouring numerous coccal shaped organisms, these however were not identified microbiologically.

Even though only *B.subtilis* was isolated from the collected algae, *E.biruncinata* and *S.heterophyllum* both showed some activity against the *Micrococcus* species, with *E.biruncinata* showing very strong activity against *S.aureus*. (Table 2.3.1 and 3.3.1) *S.heterophyllum* however showed only slight activity against *S.aureus*. Both *S.aureus* and *Micrococcus* are micrococci of size approximately 1µm in diameter. They are unicellular spheres of regular cell size which do not occur in regular groups. From the bar size given on the micrographs, it is possible that the cocci visible may be either *S.aureus* or *Micrococcus* species. The presence of these organisms in such large numbers was unexpected due to the high activities shown in Table 3.3.1.

The micrographs taken, would suggest that any compounds produced by the seaweeds for protection, are evenly distributed within the thallus, as there does not appear to be any variation in bacterial concentration between the tip, middle and base of the thallus. Further work would be necessary to confirm these results, however this is in agreement with Paul and Fenical (1986) who, on studying various Caulerpacean species, found there to be similar concentrations of metabolites within the blades, stipes and holdfasts of these algae. The allocation of various metabolites to specific tissues would appear to be unfeasible and costly to the plant. Even so, some workers have found specific distributions of activity within the thallus. Hornsey and Hide (1976) found *Ulva lactuca*, to have a uniform distribution of antibiotic activity. *Chondrus crispus*, *Dilsea carnosa* and *Codium fragile*, however, exhibited increased activity in the younger regions of the thallus, and *Laminaria saccharina* had a more complicated distribution, this being a decrease of activity in the lamina just behind the meristem, but then increased activity towards the older parts of the blade. Seasonal studies of these algae showed there to be no variations in activity.

Colon *et al.* (1987) found greater quantities of secondary metabolites in the blade region of *Avrainvillea nigricans*. This strongly suggests that these metabolites are produced specifically to play defense roles. Another example of a secondary metabolite being used as protection, is the bromophenol, lanasol obtained from *Polysiphonia lanosa* (L.) Tandy. Lanasol is toxic to microalgae grown in culture, and this would suggest, that the production of this compound is primarily to inhibit epiphytic algal encrustation. The sulphate salts of these bromophenols however have been shown to have no antialgal properties,

and in fact are mildly stimulating. Bromophenols are thought to occur naturally as sulphates, but on exposure to sea water, are hydrolyzed to the active phenols. A study on the exterior surface of the *P. lanosa* thallus however revealed high concentrations of epiphytic microflora, even though this alga is rich in bromophenols. These epiphytes may be attracted to, or be utilizing the halogenated metabolites which are being produced [Fenical, 1975]. This may be the case with *E. biruncinata*; the organisms shown in the micrographs may be utilizing the secondary metabolites produced by the alga, even though the microbiological studies showed *E. biruncinata* to be highly active against the two Gram positive cocci examined.

There appears to be a lot of discrepancy as to the distribution and function of secondary metabolites produced by algae. The general opinions however appears to be, that these compounds are produced to protect the algae against predation, and settling and encrustation by fouling organisms which would reduce the chances of survival of the alga.

## CHAPTER V

### THE ANTINEOPLASTIC ACTIVITIES OF SELECTED SEAWEED EXTRACTS

#### 5.1 Introduction:

Cancer, or the development of malignant tumours occurs when cells escape the normal regulatory processes and divide without restraint. The mass of abnormal tissue which occurs as a result of this is known as a tumour. The growth of malignant tumours is invasive, they damage the organ in which they occur ultimately causing death of the animal. Often transformed cells are released from the tumour and form new neoplastic foci. Not all tumours however are malignant. Some are benign, and so remain localized and have no harmful effect on the animal. Whereas cancer cells grow in this unregulated manner, normal tissues, in contrast, are formed by the regulated, limited growth of their component cells.

The induction of a tumour *in vivo* can be reproduced in tissue cultures [Stanier *et al.*, 1980]. The cells are transformed into a type that exhibits unregulated growth. This process of transformation causes a number of striking changes in the cells. Most animal cells in tissue culture exhibit the phenomenon of contact inhibition in which the cells move about randomly by amoeboid motion and divide repeatedly until they come into contact with one another, contact with cells therefore inhibits both movement and cell division. The result of this phenomenon is that normal cells in tissue culture form a monolayer on the surface of the vessel, whereas transformed cells do not exhibit contact inhibition, and so form tumour-like cell masses. These cells which are transformed in tissue culture will also initiate tumours when inoculated into host animals.

Cancer has been a cause of death in humans for centuries. Over the years, many varied forms of research have, and are being carried out to try and cure this condition. The latest research on anticancer drugs based on compound structures, has revealed that alkylating groups require a clearly defined three-dimensional geometry to produce antitumour activity. This geometry however may not be the same as that required to produce toxicity [Fox, 1988].

Recent studies in anticancer drug design involves a number of new ideas, one of which involves receptor mapping of bifunctional nucleophilic centres which must be attacked in order to bring about antitumour activity. This is in contrast to those compounds which result in toxicity effects in normal tissues.

There have also been new developments in anticancer drug development in the anthracycline field. Modifications have been made on both the aglycone and carbohydrate moieties of the typical anthracycline structures. DNA base preference changes occur during intercalation as a result of these changes. Assay of these drugs requires the development of new systems which have a specific base requirement for their expression. Some promising results have been observed with the selective actions of distamycin analogues, new irreversible aromatase inhibitors and dopamine antagonists.

Novel bioreductive agents in the mitosene group are also being researched. The bioreductive activation concept is potentially of major significance as a mechanism of action of many naturally occurring and synthetic antineoplastic compounds. They usually rely on the formation of reduced quinone compounds, *in vivo*. Considerably enhanced activity following reduction of a quinone moiety is found. The mitosenes are a group which are examples of these agents and are based on the structure of mitomycin C.

The aromatic azido group has been used as an anticancer agent, especially with regard to the development of some new lipophylic anticancer folates, producing new dihydrofolate reductase inhibitors. One of these, the ethane sulphonic acid salt of 2,4-diamino-5-(3-azido-4-chlorophenyl)-6-ethylpyrimidine is an important inhibitor of rat liver dihydrofolate reductase. This compound is undergoing clinical trials in the United Kingdom [Fox, 1988].

Following the successful synthesis of the fungal metabolite, sparsomycin, two new analogues with increased lipophylic properties were prepared. The active function of the molecule is the unusual monooxodithioacetal group. The two new analogues are at present being considered for clinical trials.

Another new development involves protooncogene activation. This has been implicated as being involved in the induction, maintenance and progression of human malignant phenotypes. Some of these are amplified in a number of human tumours and the possibilities of controlling the expression at mRNA level is being seriously researched. The development of antisense RNA which exerts a negative action on the RNA expression of oncogenes, especially in relation to the possible multigenic character of the malignant expression, has been found. A series of tetrathymidylates have been designed to interact covalently with the DNA intercalator head. The results obtained made possible some applications of such oligonucleotides as antisense ligands to the transcription products of some oncogenes. This is the newest area of potential anticancer drug therapy [Fox, 1988].

Even though there are many new fields of anticancer drug development, as shown above, it is essential not to lose sight of the natural products that are available and that have not yet been explored to their full potential.

Seaweeds are one group that have shown great anticancer drug potential over the past centuries and particularly in the last few decades. The Chinese have used seaweeds in the treatment of cancer for centuries. The species which were used mainly in their decoctions were *Sargassum* and *Laminaria*. Records of successful treatment by the Chinese led modern scientists to investigate the potential of this group more thoroughly. Since then, the compounds bryostatins and didemnins have entered clinical trials, as well as many others. Table 5.1.1. records some of the algae showing activity against various tumour cell lines. In this study 9 seaweed extracts were tested for antineoplastic activity by determining their growth inhibitory effects in cell culture on a mouse melanoma cell line and a normal, non-malignant cell line.

Table 5.1.1 The anticancer activity of various algae.

| Alga                                                                                                                                                                                                                                                                                                                                                | Active Compound                                                                                                                                                                                                                                                                                                                                                                                                                                              | Tumour cell type                                                                                                                                                                                                                         | Reference                                                                                                                                  |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------|
| <u>Blue-green alga</u><br>(Oscillatoriaceae:<br>Nostocales)                                                                                                                                                                                                                                                                                         | debromoaplysiatoxin                                                                                                                                                                                                                                                                                                                                                                                                                                          | lymphocytic mouse leukemia<br>1977                                                                                                                                                                                                       | Myderse <i>et al.</i> ,                                                                                                                    |
| <u>Marine cyanophyte</u><br><i>Hormothamnion</i><br><i>enteromorphoides</i> Grunow                                                                                                                                                                                                                                                                  | hormothamnione - RNA synthesis<br>inhibition                                                                                                                                                                                                                                                                                                                                                                                                                 | P388 lymphocytic leukemia<br>HL-60 human promyelocytic<br>leukemia                                                                                                                                                                       | Gerwick <i>et al.</i> ,<br>1986                                                                                                            |
| <u>Marine microalga</u><br><i>Chlamydomonas</i> sp.<br>(Volvocales)                                                                                                                                                                                                                                                                                 | L-asparaginase                                                                                                                                                                                                                                                                                                                                                                                                                                               | lymphosarcoma                                                                                                                                                                                                                            | Paul, 1982                                                                                                                                 |
| <u>Chlorophyceae</u><br><i>Sargassum</i> sp.<br><br><i>Laminaria</i> sp. (Chinese<br>herbal remedies)<br><i>Avrainvillea nigricans</i><br>Decaisne (Udoteaceae)<br><i>Anadyomene menziesii</i><br><br><i>Anadyomene stellata</i><br><br><br><i>Caulerpa prolifera</i><br><br><i>Caulerpa sertularioides</i><br><br><br><i>Caulerpa verticillata</i> | host mediated inhibition by<br>nondialyzable polysaccharide<br><br>3-bromo-4,5-dihydroxybenzyl<br>alcohol<br>aqueous extract (unidentified<br>compound)<br>chloroform extract (lipids e.g.<br>terpene derivatives)<br><br>aqueous extract (unidentified<br>compound)<br>Caulerpenyne<br>aqueous extract (unidentified<br>compound)<br>chloroform & methanol extract<br>(lipids)<br>aqueous extract (unidentified<br>compound)<br>chloroform extract (lipids) | mouse sarcoma-180<br>mouse leukemia<br><br>human epidermal carcinoma<br>(KB)<br>human epidermal carcinoma<br>(KB)<br>human epidermal carcinoma<br>(KB)<br>mouse lymphocytic leukemia<br>(PS)<br>KB & PS<br>KB & PS<br><br>PS<br>PS<br>PS | Yamamoto <i>et al.</i> ,<br>1974<br>Colon <i>et al.</i> , 1987<br>Hodgson, 1984<br>Hodgson, 1984<br><br>Hodgson, 1984<br><br>Hodgson, 1984 |
| <u>Phaeophyceae</u><br><i>Stypopodium zonale</i>                                                                                                                                                                                                                                                                                                    | chloroform & methanolic extracts<br>stypoldione                                                                                                                                                                                                                                                                                                                                                                                                              | PS<br>P388 lymphocytic leukemia cells<br>Erich Ascites tumour cells                                                                                                                                                                      | Hodgson, 1984<br>O'Brien <i>et al.</i> ,<br>1984                                                                                           |

## 5.2 Materials and Methods:

### 5.2 (a) Tissue Culture Procedure:

Cell lines: LLCMK (monkey kidney) – Highveld Biological Association, RSA. (normal cells)

Mouse melanoma-350 – Highveld Biological Association, RSA. (malignant cells)

Foetal Calf Serum (FCS) – State Vaccine Institute, RSA.

Eagle's Minimal Essential Medium (MEM) – Highveld Biological Association, RSA.

#### (i) Thawing of Cells:

Stocks of cell suspensions frozen in liquid N<sub>2</sub> were kept in readiness for use. When required, these cells were removed from the liquid N<sub>2</sub>. The vials in which they were stored were placed in a beaker of distilled water which was then placed in a water bath at 37°C. When thawed, the vial was washed thoroughly with 95% ethanol to sterilize it. Working at a laminar flow bench at all times, the contents of the vial were transferred to a 75 cm<sup>2</sup> tissue culture flask. 60 ml of sterilized media containing 15% foetal calf serum (FCS) was added to the flask. The flask was then incubated at 37°C. One change of sterile 15% FCS supplemented medium was made after 12 hrs and the flask was checked for contamination after 24 hrs. If free of contamination the medium was changed using 10% FCS supplemented medium. The cells were incubated at 37°C until a confluent monolayer was obtained. The cells were then either harvested for an experiment, or split into a number of flasks to increase the number of cells available.

#### (ii) Splitting of Cells:

To split the cells, the media was poured off and 10 ml of trypsin, filtered through a 0,45 µm filter, was added to each flask. The flasks were incubated at 37°C until the cells had lifted off the surface. The cells were then transferred to sterile centrifuge tubes in which they were centrifuged for 10 min at 1000g in a Hettich universal K2S. The trypsin was poured off, leaving the pellet of cells which were resuspended in a small amount of 10% FCS supplemented medium. The cells were distributed between 4 flasks, each containing 30 ml of sterile 10% FCS supplemented medium.

#### (iii) Harvesting of Cells and Preparation of Experimental Cultures:

Flasks containing confluent monolayers of cells to be harvested were treated similarly to those used for splitting, except that after centrifugation, the trypsin was poured off, and 5 ml of sterile 10% FCS supplemented medium was added. The cells were resuspended, then counted using a haemocytometer, to determine the cell count per ml of medium.  $0,3 \times 10^6$  cells were added to each experimental flask. After adding the correct number of cells to each flask, 10 ml of prepared sterile 10% FCS supplemented medium was added to each flask, as well as the prepared algal extracts in varying quantities.

### 5.2 (b) Algal Extracts:

The nine seaweeds collected on the 5/3/87 at the rocky outcrop called the First Sister near Riet River, were used for this study. The seaweeds used are listed in Section 2.2. The algae were extracted in methanol then dried using a rotary evaporator, after which they were resuspended in a small volume of

95% ethanol to make up a 500% solution i.e. 500 g extract\100 ml. 95% ethanol was also used as the control. The extracts were filtered through 0,45µm filters before being used.

#### 5.2 (c) In vitro Experiment:

Two cell lines were used for this experiment, the normal cell line, LLCMK (monkey kidney) and the malignant cell line, mouse melanoma-350. A comparison was drawn on the effects that the algal extracts had on these two cell-lines.

Initially an experiment was carried out using 200µl of algal extract per flask of cells. In all subsequent experiments, 0, 10, 25 or 50 µls of extract was added, six replicas were made for each treatment. The flasks were incubated at 37°C until the cells of the control flasks reached confluency. The control flasks (0 µl extract) had 50µl of 95% ethanol added only.

#### 5.2 (d) In vivo Experiment:

White female hairless mice, HRS\J strain, obtained from Jackson Laboratories, USA, were injected subcutaneously in the stomach region with  $0,3 \times 10^6$  melanoma-350 cells suspended in 1 ml phosphate buffer (pH 7,5 0,1M). Experimental diets were fed immediately after injection, with six mice being used per group. These mice were fed a standard balanced diet and in addition were fed orally, once daily with 50, 100 or 200 µl of prepared algal extract suspended in phosphate buffer (pH 7,5, 0,1M). Treatment continued for 6 weeks, with deaths being recorded, and the condition of the animals noted.

#### 5.3 Results and Discussion:

Results are expressed as % cell growth relative to the control cultures (100%). The initial experiment using 200 µl of extract per flask of mouse melanoma-350 and LLCMK cells was used to determine the toxic volume of alga per flask. This experiment showed, that all three of the brown algae tested resulted in 100% inhibition of cell growth at a volume of 200µl. This however was not the case for the red and green algae. Of the green algae, *Caulerpa filiformis* caused 100% inhibition of cell growth, with *Ulva rigida* causing 85% and *Codium duthiae* 63% inhibition. The red algae were more inconsistent, with *Amphiroa tenera* and *Hypnea spicifera* resulting in a reduction in cell number, and *Spyridia cupressina* stimulating cell growth at this concentration. As a result of the data from this experiment it was decided to use a lower volume of extract, consequently 10, 25 and 50µl of algal extract was used per flask in all subsequent experiments.

The effects that the seaweed extracts had on both the normal and malignant cell lines used are represented in Figures 19-27. The vertical bars represent the standard deviation from the mean % cell growth.

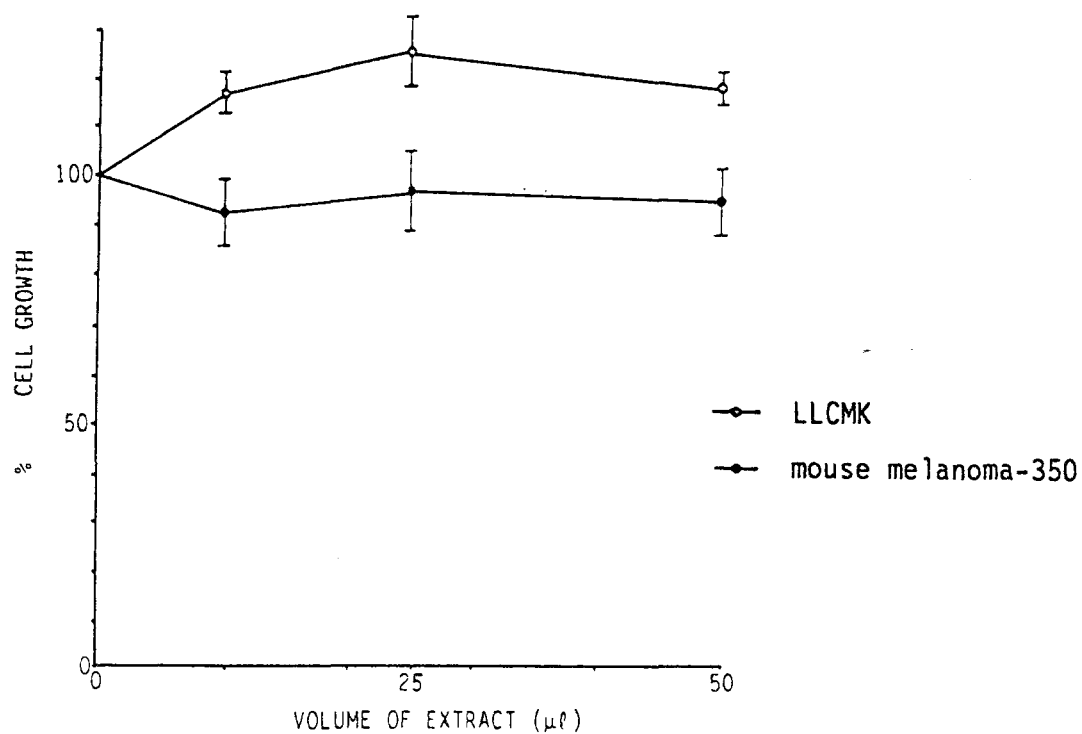


Fig. 19 The effect of the *Amphiroa anceps* extract on LLCMK (monkey kidney) and mouse melanoma-350 cells.

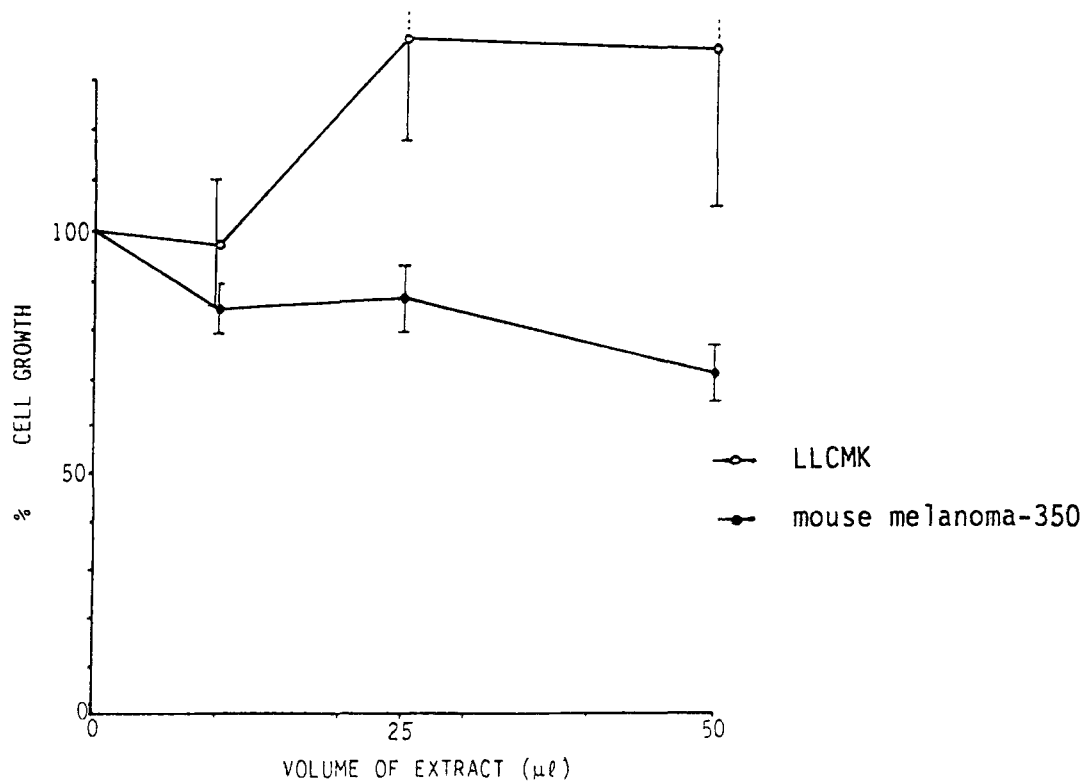


Fig. 20 The effect of the *Spyridia cupressina* extract on LLCMK (monkey kidney) and mouse melanoma-350 cells.

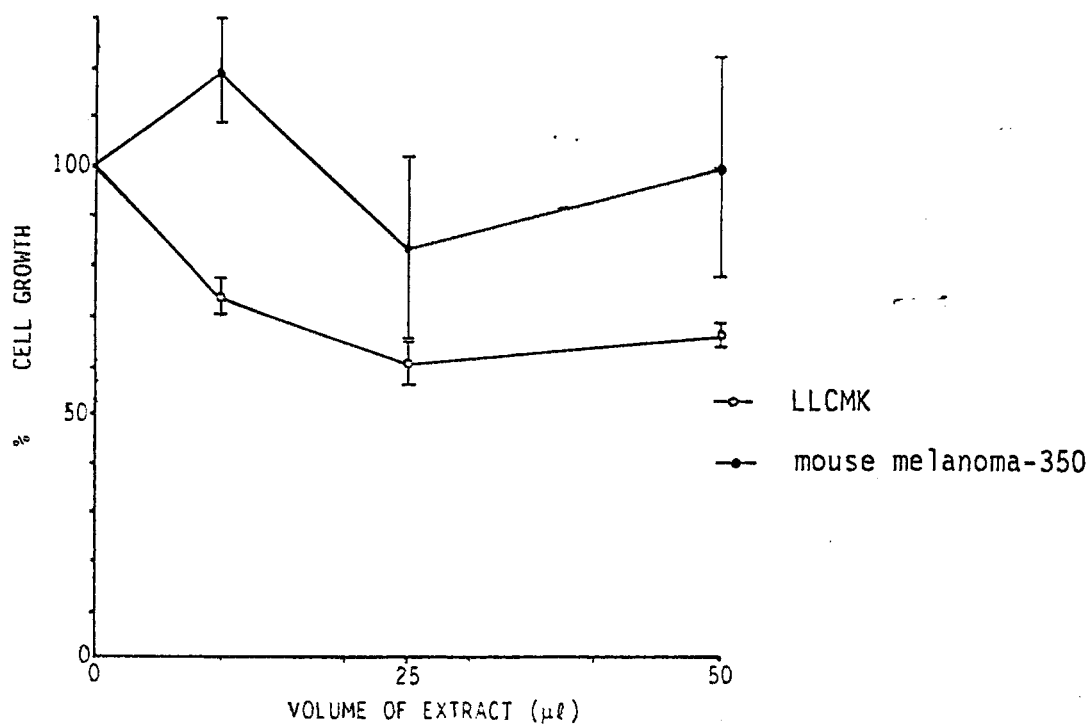


Fig. 21 The effect of the *Hypnea spicifera* extract on LLCMK (monkey kidney) and mouse melanoma-350 cells.

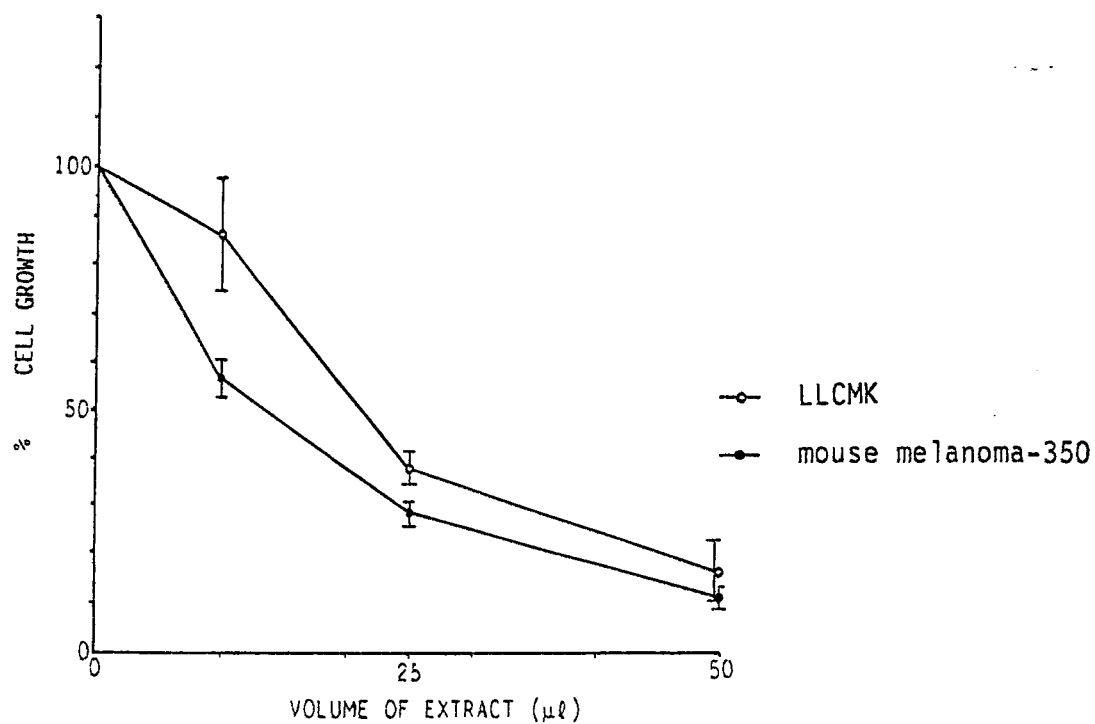


Fig. 22 The effect of the *Zonaria subarticulata* extract on LLCMK (monkey kidney) and mouse melanoma-350 cells.

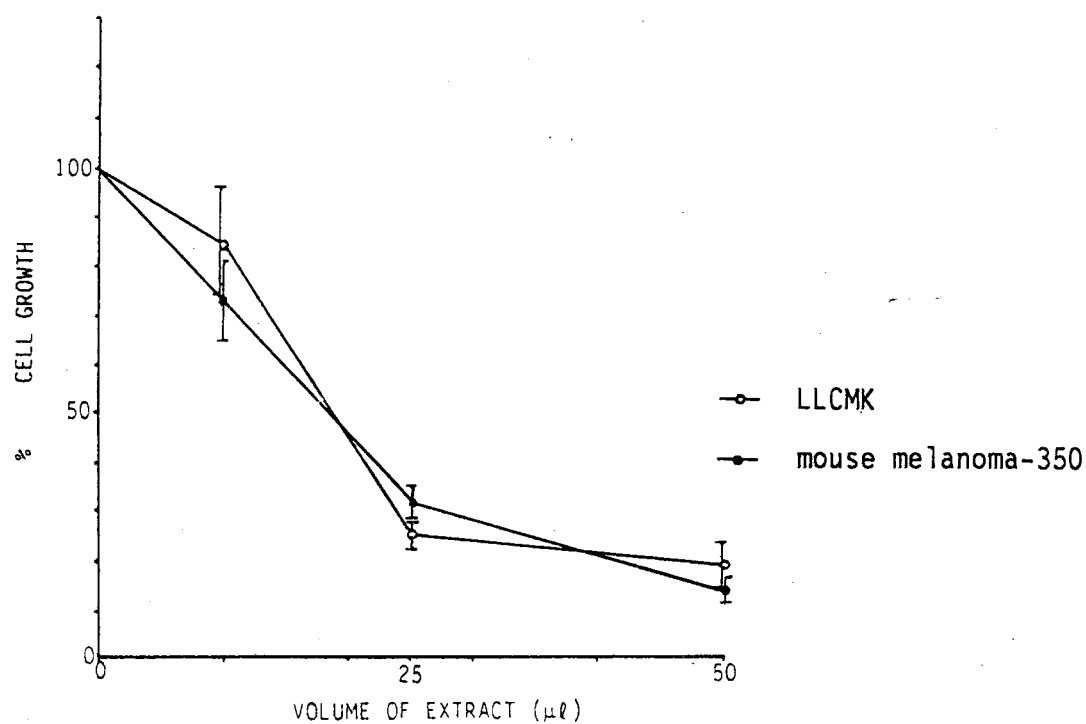


Fig. 23 The effect of the *Ecklonia biruncinata* extract on LLCMK (monkey kidney) and mouse melanoma-350 cells.

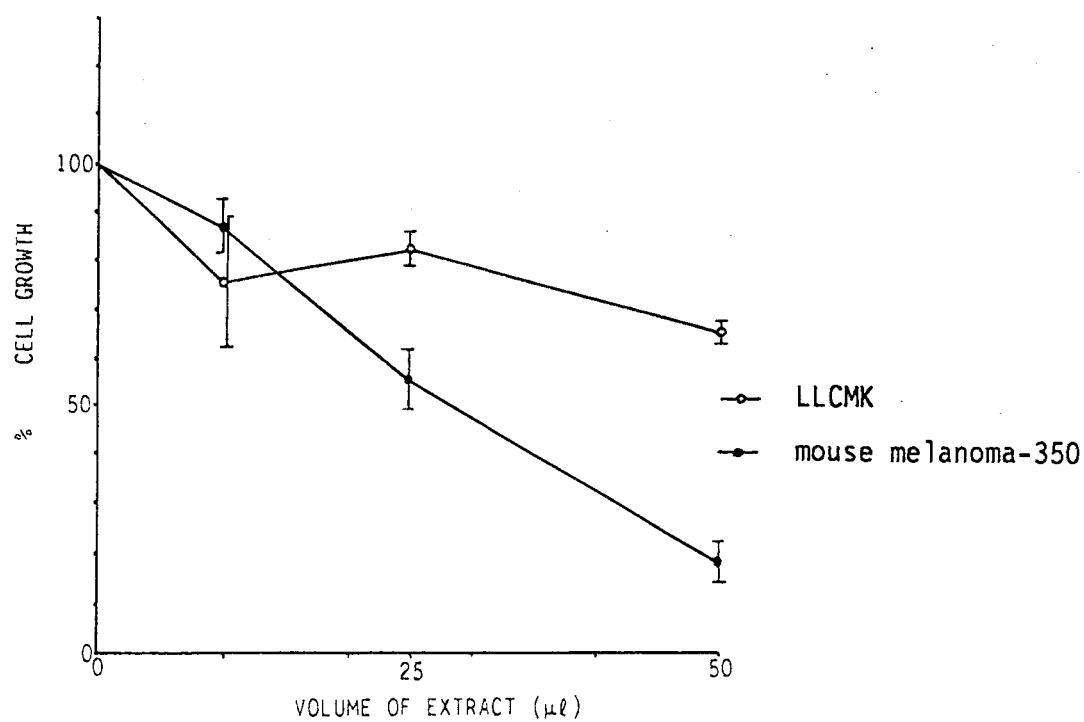


Fig. 24 The effect of the *Sargassum heterophyllum* extract on LLCMK (monkey kidney) and mouse melanoma-350 cells.

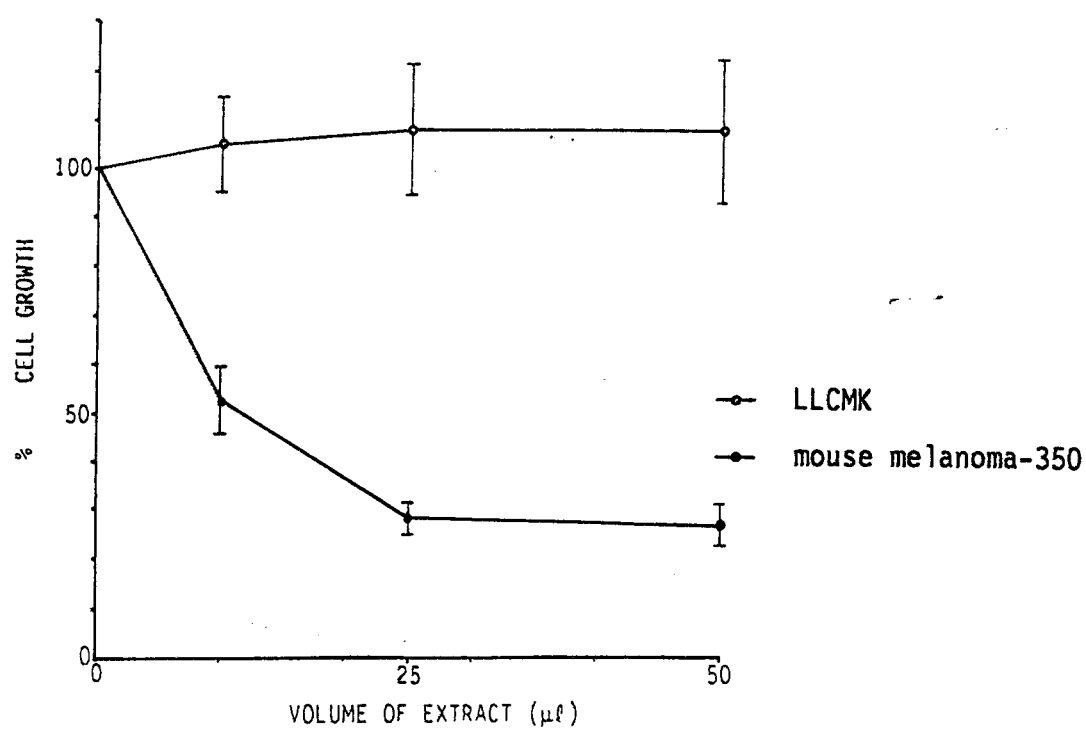


Fig. 25 The effect of the *Ulva rigida* extract on LLCMK (monkey kidney) and mouse melanoma-350 cells.

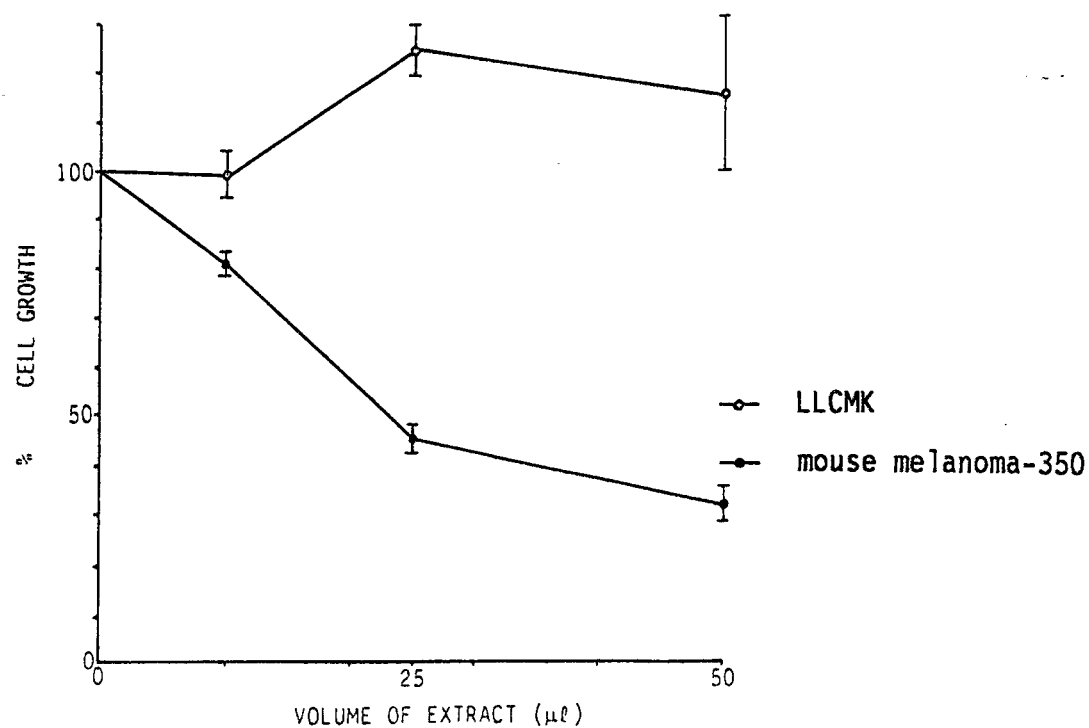


Fig. 26 The effect of the *Caulerpa filiformis* extract on LLCMK (monkey kidney) and mouse melanoma-350 cells.

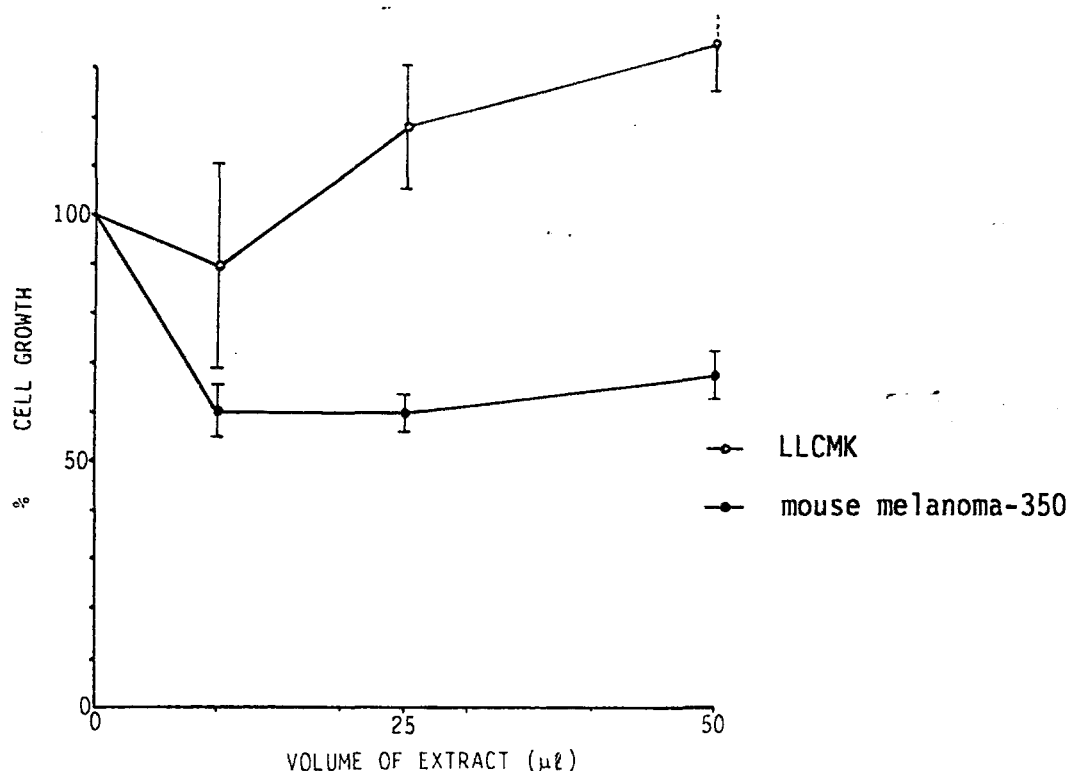


Fig. 27 The effect of the *Codium duthiae* extract on LLCMK (monkey kidney) and mouse melanoma-350 cells.

The three red algae did not substantially reduce the growth of either the LLCMK or the mouse melanoma-350 cells, however *Spyridia cupressina* stimulated LLCMK growth and mildly reduced the growth of the cancerous cells.

The brown algae on the other hand had a dramatic effect on cell growth. *Sargassum heterophyllum* reduced LLCMK growth to 66% of controls, and mouse melanoma-350 growth to 17% of controls at an extract volume of 50 μl. It is worth noting, that the normal cells grew better at this concentration of algal extract than did the cancerous cells. The other two brown algae, namely *Zonaria subarticulata* and *Ecklonia biruncinata* reduced the growth of both the normal and cancerous cell lines equally to a range of 11-19% of the growth of the controls at 50μl of extract. This is an undesirable effect, as in the treatment of cancerous conditions, it is essential that only the cancerous cells are affected by the treatment and that the normal cells continue functioning normally. The brown algae contain large quantities of phenols which may be responsible for these results, as they are known to bind and inactivate proteins and enzymes. They are therefore toxic to all cells [Lobban *et al.*, 1985].

The seaweeds which gave particularly good results were the three members of the Chlorophyceae group that were studied. The *Ulva rigida* and *Caulerpa filiformis* extracts both stimulated the growth of the LLCMK cells slightly and reduced the growth of the mouse melanoma-350 cells substantially i.e. to 27% and 33% of controls respectively at 50μl of extract. *Codium duthiae* had less dramatic effects stimulating the LLCMK cell growth to 135% of controls but only reducing mouse melanoma-350 growth to 68% of controls at 50μl of extract.

Due to the results obtained in the *in vitro* study of the effects of selected seaweeds on LLCMK and mouse melanoma-350 cell growth, the study was continued using mouse melanoma-350 tumour cells injected into mice. The three algal extracts which most substantially affected *in vitro* melanoma growth, *U.rigida*, *C.filiformis* and *Z.subarticulata*, were fed orally to the mice as described. The reason for administering the treatment orally was that this is the favoured method for administering drugs to patients, besides being most convenient, it also required less time and hence reduced the cost of treatment. Oral administration was therefore used. It was also necessary to determine whether the drug was absorbed through the digestive system or not. If the active ingredient became altered in any way before absorption and hence became inactive, an alternative method of administration would have been required.

Unfortunately this experiment was unsuccessful since the tumours did not grow in either the control mice or the experimental mice. Subsequent problems experienced with getting these cells to grow *in vivo* meant that it was not possible to repeat the experiment. The *Z.subarticulata* extract however did appear to be toxic to mice. Three deaths were recorded, and the remainder of the mice showed weight losses, and deterioration in coat condition. The *U.rigida* and *C.filiformis* treated mice remained healthy and in good condition, and no deaths were recorded.

CHAPTER VI  
BRINE SHRIMP ASSAY FOR ACTIVE CONSTITUENTS

6.1 Introduction:

Many new natural products are isolated, characterised, and their chemical structures published without any investigation of the potential biological activity of these compounds. Accordingly, their biological potential is never realized. Until biological data is obtained for these compounds, they are of no medicinal value. One problem with biological testing, is that often the bioassays employed are costly and are too specific, with the result that useful activities are often not detected in the screening procedure. These tests also take considerable periods of time to perform. Common toxicity assays include the

- (i) sea urchin fertilized egg cytotoxicity assay,
- (ii) sea urchin sperm assay,
- (iii) sea urchin larval toxicity assay and
- (iv) fish toxicity assay [Paul and Fenical, 1986].

These tests require experts to perform them, and to maintain the necessary organisms and equipment. There is therefore a need for a rapid, reliable and simple general bioassay which can be run at low cost to detect a broad spectrum of pharmacological activities.

Bioactive compounds are almost always toxic in high doses. It has been found however, that a simple crustacean, the brine shrimp *Artemia salina* Leach can be used to screen new biologically active natural products. The brine shrimp lethality test is advantageous, as large numbers of organisms can be used for statistical validations, it requires no special equipment and relatively small quantities of samples. Brine shrimp eggs are also readily available and can be obtained at pet shops at very low cost. They require no maintenance, and can be stored for years without losing their viability. Following immersion in sea water, the nauplii will hatch within 24-48 hrs. The brine shrimp assay therefore has an added advantage over other bioassay techniques, in that the eggs require no stock culturing. Table 6.1.1 summarizes some of the previous uses of brine shrimp as a bioassay. The assay system was consequently tested in this study as a means of identifying the biological activity of seaweed extracts.

Table 6.1.1 The uses of brine shrimp in general bioassays.

| Applications: analysis of : –                 | Reference                                                                                                                            |
|-----------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------|
| pesticide residues                            | Michael <i>et al.</i> , 1956<br>Tarpley, 1958<br>Areekul and Harwood, 1960<br>Grosch, 1967                                           |
| mycotoxins                                    | Brown <i>et al.</i> , 1968<br>Brown, 1969<br>Harwig and Scott, 1971<br>Eppley, 1974<br>Korpinen, 1974<br>Eng-Wilmot and Martin, 1979 |
| stream pollutants                             | Hood <i>et al.</i> , 1960                                                                                                            |
| anaesthetics                                  | Robinson <i>et al.</i> , 1965                                                                                                        |
| dinoflagellate toxins                         | Granade <i>et al.</i> , 1976                                                                                                         |
| morphine-like compounds                       | Richter and Goldstein <i>et al.</i> , 1970                                                                                           |
| toxicity of oil dispersants                   | Zillioux <i>et al.</i> , 1973                                                                                                        |
| cocarcinogenicity of phorbol esters           | Kinghorn <i>et al.</i> , 1967                                                                                                        |
| toxicants in marine environments              | Vanhaecke <i>et al.</i> , 1981                                                                                                       |
| higher plants: Euphorbiaceae seeds            | Meyer <i>et al.</i> , 1982                                                                                                           |
| cytotoxic and murine leukemia active extracts | McLaughlin and Anderson, 1989                                                                                                        |

## 6.2 Materials and Methods:

### 6.2 (a) Algae:

Four algae were used in this brine shrimp (*Artemia salina*) cytotoxicity study. These were:

- (i) Red Alga *Amphiroa anceps*
- (ii) brown alga *Zonaria subarticulata*
- (iii) green alga *Ulva rigida*
- (iv) green alga *Caulerpa filiformis*

These algae were chosen because of their cytotoxicity towards LLCMK (normal) and mouse melanoma-350 (cancer) cells. See Section 5.3, Figures 19, 22, 25 and 26. The red alga *A.anceps* only marginally reduced the growth of the cancerous cells, and appeared to stimulate normal cell growth, whereas the brown alga *Z.subarticulata* reduced all cell growth dramatically. The two green algae however stimulated normal cell growth to varying degrees, while reducing cancerous cell growth to very low levels.

#### 6.2 (b) Sample Preparation:

Methanolic extracts of the algae were diluted to 10, 100 and 1000  $\mu\text{l ml}^{-1}$  concentration with methanol. The appropriate amounts of solution were transferred to filter paper discs which were air dried, and then placed in vials. The control discs were prepared using methanol only. Five replicates of each preparation were made.

#### 6.2 (c) Hatching of Brine Shrimp:

Brine shrimp eggs were hatched in a shallow rectangular container filled with natural sea water which had been autoclaved, filtered and aerated. A plastic divider in which several 2mm holes had been made was placed in the dish to make two unequal compartments. The eggs were placed in the larger compartment which was kept dark, while the smaller compartment was illuminated. Within 48 hrs the phototropic nauplii (larvae) had moved through the divider, leaving the egg shells behind. These nauplii were then removed from the illuminated section with a pipette when required for each assay.

#### 6.2 (d) Bioassay:

5 ml of seawater was placed into each vial together with the filter paper disc loaded with the algal extract. Ten brine shrimp nauplii were introduced into each vial, after which 2 drops of the microalga, *Tetraselmis seucica*, suspension was added as food. The vials were maintained under illumination, and the survivors were counted after 24, 27 and 30 hrs.

#### 6.3 Results and Discussion:

No deaths of the brine shrimp were recorded after 24, 27 and 30 hr at a concentration of 1000  $\mu\text{l ml}^{-1}$ , therefore the seaweed extracts tested were non-toxic to brine shrimp ( $\text{LC}_{50} > 1000 \mu\text{g ml}^{-1}$ )

The brine shrimp *Artemia salina* is a convenient organism to work with. The bioassay is simple to perform, and requires little expertise. However Sorgeloos *et al.* (1978) have indicated that reproducibility of results depends on various factors, including:

- (i) the origin of the strain of brine shrimp. The various strains require different times to complete embryonic development. The San Francisco Bay strain (California, USA) was used, and required 18 hr to achieve a 50% hatch. The first instar nauplii of the various strains of brine shrimp also display varying sensitivities to toxicants. The San Francisco Bay and Burgas-Pomorije strains showed high sensitivities, with the Tsingtao and Great Salt Lake brands being less sensitive.
- (ii) temperature during incubation and hatching. This was kept constant.
- (iii) the moment of harvesting of larvae – constant.
- (iv) the period of time between harvest and the start of the bioassay. The larvae appear to have varying degrees of sensitivity at different stages of morphological development (first, second and third instar). As the second and third instars were found to be significantly more sensitive to toxicants than first instar larva, it was important that nauplii of exactly the same age were used in every experiment. Second and third instar nauplii were used.
- (v) temperature of medium during testing – constant.
- (vi) salinity of medium during testing – constant.

From the results obtained, it is evident that the four algae tested are clearly non-toxic to the brine shrimp *Artemia salina* Leach, San Francisco Bay strain.

## CHAPTER VII

### ANTIVIRAL ACTIVITIES OF SELECTED SEAWEED EXTRACTS

#### 7.1 Introduction:

The use of algae for the prevention and cure of various diseases and ailments is widespread in certain regions of the world. The treatment of bacterial and fungal infections, burns, coughs, open wounds and fevers are commonly known [Stein & Borden, 1984]. However, the treatment of viral infections with algal and other marine preparations has not been widely reported. This aspect of biomedical research has been only superficially explored, hence the true antiviral potential of the marine flora and fauna has not yet been exploited. Organisms that have been found to contain antiviral substances as well as the nature of the compounds responsible for this action are reported in Table 7.1.1.

Table 7.1.1 Antiviral activity from compounds of marine origin.

| Origin of Compound                                                                                 | Type of Compound                                                                                                                             | Active Against                                                                                                              | Reference                                                 |
|----------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------|
| <u>Sea Sponge</u><br><i>Cryptotethya crypta</i>                                                    | nucleosides; spongothymidine<br>spongouridine & spongosine<br>(served as models for D-arabinosyl cytosine & adenine<br>arabinoside (Ara-A))  | Ara-A commercially used                                                                                                     | Bergmann & Feeney, 1950,<br>1951                          |
| <u>Algae</u><br><u>Rhodophyceae</u><br><i>Order: Cryptonemiales</i><br><i>Constantinia simplex</i> | polysaccharide<br><br>treatment before viral inoculation of cells or treatment<br>2 hr before viral inoculation of mice at site of infection | Herpes Simplex virus(HSV)-1, HSV-2<br>Coxsackie B <sub>5</sub> -virus<br>Vaccinia virus<br>vesicular stomatitis virus (VSV) | Ehresmann <i>et al.</i> ,1977                             |
| <i>Cryptosiphonia woodii</i><br>(J.Agardh) J.Agardh                                                | polysaccharide                                                                                                                               | HSV-1,HSV-2<br>HSV-1,HSV-2                                                                                                  | Richards <i>et al.</i> , 1978<br>Deig <i>et al.</i> ,1974 |
| <i>Endocladia muricata</i>                                                                         | polysaccharide                                                                                                                               | HSV-1,HSV-2                                                                                                                 | Ehresmann <i>et al.</i> ,1977                             |
| <i>Farlowia mollis</i><br>(Harvey& Bailey)                                                         | polysaccharide                                                                                                                               | HSV-1,HSV-2                                                                                                                 | Deig <i>et al.</i> ,1974                                  |
| Farlow & Setchell                                                                                  | (treatment before viral inoculation of cells or treatment<br>2 hr before viral inoculation of mice at site of infection                      | Vaccinia virus<br>VSV                                                                                                       | Richards <i>et al.</i> ,1978                              |
| <i>Farlowia crassa</i>                                                                             | polysaccharide                                                                                                                               | HSV-1,HSV-2                                                                                                                 | Ehresmann <i>et al.</i> ,1977                             |
| <i>Farlowia compressa</i>                                                                          | polysaccharide                                                                                                                               | HSV-1,HSV-2                                                                                                                 | Ehresmann <i>et al.</i> ,1977                             |
| <i>Pikea pinnata</i>                                                                               | polysaccharide                                                                                                                               | HSV-1,HSV-2                                                                                                                 | Ehresmann <i>et al.</i> ,1977                             |
| <i>Leptocladia conferta</i>                                                                        | polysaccharide                                                                                                                               | HSV-1,HSV-2                                                                                                                 | Ehresmann <i>et al.</i> ,1977                             |

|                                                            |                                                                                                                                                                                                               |                                                                                     |                                |
|------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|--------------------------------|
| Order: Ceramiales                                          |                                                                                                                                                                                                               |                                                                                     |                                |
| <i>Cryptopleura violacea</i>                               | polysaccharide                                                                                                                                                                                                | HSV-1, HSV-2                                                                        | Ehresmann <i>et al.</i> , 1977 |
| <i>Nienburgia andersoniana</i>                             | polysaccharide                                                                                                                                                                                                | HSV-1, HSV-2                                                                        | Ehresmann <i>et al.</i> , 1977 |
| <i>Laurencia paniculata</i> J. Ag.                         | lipid<br>(unusual lipophylic metabolites)                                                                                                                                                                     | Tobacco Mosaic virus (TMV)                                                          | Caccamese <i>et al.</i> , 1980 |
| <i>Laurencia venusta</i>                                   | thysiferol and venustatriol: tetracyclic ethers of<br>triterpenoid origin<br>thysiferol-23-acetate                                                                                                            | VSV, HSV-1                                                                          | Sakemi <i>et al.</i> , 1986    |
| Order: Nemalionales                                        |                                                                                                                                                                                                               |                                                                                     |                                |
| <i>Gelidium cartilagineum</i>                              | polysaccharide composed primarily of D-galactose<br>units (Source of agar)                                                                                                                                    | Influenza B<br>mumps virus (inactive against Influenza A & Newcastle Disease Virus) | Gerber <i>et al.</i> , 1958    |
| <i>Chondus crispus</i>                                     | polysaccharide composed primarily of D-galactose<br>units (Source of carrageenin)                                                                                                                             | Influenza B<br>mumps virus (inactive against Influenza A & Newcastle Disease Virus) | Gerber <i>et al.</i> , 1958    |
| <u>Phaeophyceae</u>                                        |                                                                                                                                                                                                               |                                                                                     |                                |
| Order: Dictyotales                                         |                                                                                                                                                                                                               |                                                                                     |                                |
| <i>Dictyota dichotoma</i><br>var. intricata (C. Ag.) Grev. | lipid - unusual lipophylic metabolite                                                                                                                                                                         | TMV                                                                                 | Caccamese <i>et al.</i> , 1980 |
| <i>Dilophys fasciola</i><br>(Roth) Howe                    | lipid - unusual lipophylic metabolite                                                                                                                                                                         | TMV                                                                                 | Caccamese <i>et al.</i> , 1980 |
| <i>Nereia filiformis</i><br>(J. Ag.) Zanard                | lipid - unusual lipophylic metabolite                                                                                                                                                                         | TMV                                                                                 | Caccamese <i>et al.</i> , 1980 |
| <i>Taonia atomaria</i><br>(Woodw.) J. Ag.                  | lipid - unusual lipophylic metabolite                                                                                                                                                                         | TMV                                                                                 | Caccamese <i>et al.</i> , 1980 |
| <i>Cystosiera crinita</i><br>(Desf.) Bory                  | lipid - unusual lipophylic metabolite                                                                                                                                                                         | TMV                                                                                 | Caccamese <i>et al.</i> , 1980 |
| Order: Laminariales                                        |                                                                                                                                                                                                               | Influenza virus                                                                     | Kathan, 1965                   |
| <i>Ecklonia species</i>                                    | glycoprotein<br>(inhibits neuraminidase, prevents attachment of<br>influenza virus. Protective to embryonated eggs as<br>late as 6 hr post infection. Inhibits viral hemagglutinins<br>and infectivity titre) |                                                                                     |                                |
| <u>Chlorophyceae</u>                                       |                                                                                                                                                                                                               |                                                                                     |                                |
| Order: Caulerpaceae                                        |                                                                                                                                                                                                               |                                                                                     |                                |
| <i>Caulerpa prolifera</i><br>(Forssk.) Lamour              | lipid - unusual lipophylic metabolite                                                                                                                                                                         | TMV                                                                                 | Caccamese <i>et al.</i> , 1980 |

The viruses shown in Table 7.1.1 are not the only viruses that have been tested. Other viruses that were tested by Ehresmann *et al.*, (1977) included strains of adeno 7, ECHO 6, Japanese encephalitis, polio 2, respiratory syncytical, rhino 2, Sindbis and western equine encephalitis virus. No activity was reported against these viruses. Similarly, no activity was reported against encephalomyocarditis, Semliki Forest and murine cytomegalovirus [Richards *et al.*, 1977].

## 7.2 Materials and Methods:

### 7.2 (a) Tissue Culture Procedure:

Vero cells were grown to monolayer in Minimal Eagle's Medium, (MEM) containing 5% Foetal Calf Serum (FCS) in 75 cm<sup>2</sup> tissue culture flasks (Falcon). Once confluent, the cells were either used in that form or they were trypsinized for use in virus titrations. If required for titre determinations, after the cells had lifted, 10 ml of 5% FCS- supplemented MEM was added to each flask. One flask was used per microtitre plate.

### 7.2 (b) Virus Propagation:

Polio virus Type 1 Strain L-Sa obtained from the National Institute of Virology (NIV) was grown by inoculating a 75 cm<sup>2</sup> flask of confluent Vero cells with 200µl of Polio virus to which 5ml of serum-free met<sup>+</sup> leu<sup>+</sup> MEM had been added.

The cells were incubated at 37°C on a shaker overnight. If the virus showed obvious cytopathic effect, when visualized under a microscope the cells were refrigerated for approximately 24 hrs to release the virus. The contents of the flask were centrifuged at 3000 r.p.m. in a MSE bench top centrifuge for 10 minutes to remove the cell debris. The virus-containing supernatant was then decanted into sterile bottles, and refrigerated until required.

### 7.2 (c) Algal Extracts:

These were prepared as in Section 3.2.

### 7.2 (d) Experimental Procedure:

Four 25 cm<sup>2</sup> flasks of confluent Vero cells were required for each extract to be tested. The culture medium was poured off, and the cells were rinsed with serum-free met<sup>+</sup> leu<sup>+</sup> MEM. 5ml of serum-free MEM was then added to each flask, after which the cells were treated in the following way:

- (i) 50 µl virus (prepared as in section 7.2 (b)) added only
- (ii) 50 µl virus + 200µl (or 100µl) algal extract added
- (iii) 200µl (or 100µl) algal extract only
- (iv) cells only i.e. no additions

The treated cells were incubated for approximately 24 hrs on a shaker at 37°C. They were then checked visually under a microscope to confirm that the untreated cells were growing normally, and to check whether the algal extracts were cytotoxic or not. If cytotoxic the algal extracts caused the Vero cells to lift off the flask surfaces.

If the cells had grown normally, and the algal extracts were non-toxic, the treatments (i) and (ii) were refrigerated for approximately 24 hrs to release the virus, after which the contents were centrifuged to remove cell debris. The virus solutions were then decanted, and 10-fold dilution series prepared of both treatments in serum-free MEM.

#### 7.2 (e) Virus Titre Determination:

The virus titre was determined using a 96-well flat-bottomed microtitre plate, manufactured by NUNC, to which various quantities of virus, cells and media were added.

It was necessary to determine the titre of the virus preparation alone (treatment i), as well as the titre of the virus and algal preparation (treatment ii). This was done by preparing a microtitre plate for each treatment in the following way:

Rows 1-10, add

100µl of 10% FCS supplemented MEM per well

50µl of each virus dilution per well

i.e. row 1 use 50µl  $10^{-1}$  dilution

row 2 use 50µl  $10^{-2}$  dilution etc.

50µl Vero cells per well

Rows 11 and 12 (controls), add

100µl of 10% FCS supplemented MEM per well

50µl serum-free MEM per well

50µl Vero cells per well

The plates were incubated at 37°C in a humidified atmosphere containing CO<sub>2</sub>. The cells were checked daily under the microscope until the control cells (Rows 11 and 12) reached monolayer. The plates were then fixed and stained. To fix, the plates were submerged for approximately 5 hrs in 0.01 M phosphate buffered saline (PBS) with Formalin, pH, 7.3; then rinsed lightly in water. The cells were then stained with crystal violet in ethanol for approximately 2 hrs, rinsed and dried.

The healthy cells remained attached to the flat-bottomed wells, and so were stained blue, the dead cells however were washed off, and therefore the wells remained colourless. Obtaining a result therefore involved recording the number of wells containing cells i.e. stained area, for each virus dilution, after which it was possible to calculate the virus titre. The antiviral activity of the algal extracts was measured by the reduction in virus infectivity, which was determined by comparing the virus titre obtained with algal-extract treated cells, to that obtained with untreated control cells.

#### 7.3 Results and Discussion:

In screening for antiviral activity, methanolic extracts of 12 algae were examined. Initially 200µl of extract was used per flask, and under these experimental conditions, many were found to be toxic to Vero cells, i.e. they caused the detachment of cells into the medium, and so could not be examined for antiviral activity. Extracts from the following algae were found to be toxic to Vero cells.

Table 7.3.1 Algae yielding extracts toxic to Vero cells.

| Group        | Alga                                                                                                                                                                           |
|--------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Rhodophyceae | <i>Hypnea spicifera</i><br><i>Gelidium amansii</i><br><i>Gelidium pristoides</i><br><i>Peyssonelia capensis</i><br><i>Plocamium corallorhiza</i><br><i>Laurencia glomerata</i> |
| Phaeophyceae | <i>Dictyota naevosa</i><br><i>Endarachne binghamiae</i><br><i>Iyengaria stellata</i>                                                                                           |

As so many of the algae were found to be toxic, some of the extracts were re-examined using half of the volume of extract i.e. 100 $\mu$ l. All were retested (excluding *P.corallorhiza*, *E.binghamiae* and those already found to be non-toxic.) The virus titre reductions (in logs) were recorded for those extracts found to be non-toxic.

Table 7.3.2 The virus titre reductions for the non-toxic algal extracts.

| Group         | Alga                           | Vol of Extract ( $\mu$ l) | Virus titre (logs) |
|---------------|--------------------------------|---------------------------|--------------------|
| Rhodophyceae  | <i>Hypnea spicifera</i>        | 100                       | 1.2                |
|               | <i>Laurencia glomerata</i>     | 200                       | 0.0                |
| Phaeophyceae  | <i>Dictyopteris macrocarpa</i> | 200                       | 1.9                |
|               | <i>Dictyota naevosa</i>        | 100                       | 4.0                |
| Chlorophyceae | <i>Ulva rigida</i>             | 200                       | 3.6                |
|               | <i>Halimeda cuneata</i>        | 200                       | 2.6                |

When compared with the reduction in virus titre recorded by Ehresmann *et al.*, (1977) for Herpes simplex virus, the figures shown in Table 7.3.2 appear to be highly significant, particularly those of *D.naevosa* and *U.rigida*.

From the above results, it appears that extracts from the Phaeophyceae and Chlorophyceae from this collection site have greater antiviral potential than the Rhodophyceae. This however may be an inaccurate assumption due to the small sample size. Other workers have found many members of the Rhodophyceae to exhibit strong antiviral activity (Table 7.1.1).

## CONCLUSION

A substantial amount of work has been carried out in many parts of the world on the bioactive properties of seaweeds, however South Africa has not been foremost in these studies. This study was therefore initiated to try and assess some of the seaweeds of South Africa on a similar basis to those of the rest of the world.

In attempting to fill this gap, a number of aspects were dealt with. Firstly it was necessary to establish which solvent(s) were able to extract the antimicrobially active compounds most successfully from seaweeds. A variety of solvents have been used by other workers, with no explanation as to the reasons for their use. An example of this are the solvents used for the extraction of anticancer agents, these include water, methanol, methanol/toluene (3:1), ethanol, chloroform, diethyl ether and ethyl acetate. Similarly, water, methanol/toluene (3:1), ethanol and acetone were all used for the extraction of antiviral agents. As a result of this, a comparative study using seven different solvents was carried out, and it was established, that the polar solvent, methanol proved to be the most successful, and was hence used for all further studies. Once this had been established, a number of studies were carried out to determine whether the presence of the bioactive secondary metabolites within the seaweeds varied seasonally, whether any of the 17 seaweeds studied were particularly active against the microorganisms tested and whether any particular class or order of seaweeds generally appeared to possess bioactive properties. Other workers that have dealt with seasonal variations in the production of antimicrobial substances have found four major patterns to exist, uniform production throughout the year eg. *Polysiphonia lanosa*, a winter peak of activity eg. *Ulva lactuca* and *Laurencia pinnatifida*; a summer peak eg. *Dictyota dichotoma* and a spring peak of activity eg. *Codium fragile*. In South Africa, *Ulva rigida* showed no activity at all in winter, with slight activity in autumn and spring, this is in agreement with *Ulva linza* studies by Pratt *et al.*, (1951). *Laurencia glomerata* showed reasonably good antimicrobial activity which maximized in winter. Reduced activity was recorded in spring and autumn, this is partially in agreement with Almadozar (1964). *Dictyota naevosa* showed uniform activity throughout the year, although this was minimal at all times. This result is not in agreement with Hornsey and Hide (1976) who recorded a summer peak of antimicrobial production for *Dictyota dichotoma*.

It appeared that most of the seaweeds showed no noticeable seasonal changes in bioactivity during the study period, however *Plocamium corallorhiza* did show a slight spring peak of activity, and *Laurencia glomerata* a slight winter peak. Individual seaweeds did show noteworthy activity against various microorganisms. Of the Rhodophyceae *Plocamium corallorhiza* and *Laurencia glomerata* showed broad spectrum antimicrobial activity with *L.glomerata* being the only member of the Rhodophyceae to inhibit all the Gram positive and Gram negative bacteria tested. *P.corallorhiza* and *Peyssonnelia capensis* were the only two seaweeds in this group to inhibit the growth of the yeast *Candida albicans* in any way. Within the Phaeophyceae *Zonaria subarticulata* and *Ecklonia biruncinata* showed good antimicrobial activity. *E.biruncinata* showed broad spectrum activity against all the bacteria, but was inactive against *C.albicans*, whereas *Z.subarticulata* showed the most potent activity. Of the Chlorophyceae, the activity of *Ulva rigida* against *C.albicans* was worth noting. When these seaweeds were grouped into their various orders, the order Dictyotales was found to house the most active species, followed by the

Laminariales, Gigartinales and the Ceramiales. Studies by Caccamese and Azzolina (1979), Caccamese *et al.* (1985) and Pesando and Caram (1984) also showed the Dictyotales to contain the highest concentration of biologically active species. These studies showed the Fucales to have the next highest concentration of biologically active species followed by Ceramiales, Gigartinales and Cryptonemiales. The differences between these results and those obtained in South Africa may well be due to the small sample sizes used in the eastern Cape study.

It was also necessary to assess the varying susceptibility of the test microorganisms to treatment with the seaweed extracts. It became apparent, that the microorganisms do differ greatly in their susceptibilities to treatment, with *Staphylococcus aureus* being the most sensitive organism followed by the *Micrococcus* species. The yeast *Candida albicans* proved to be the most resistant organism with only three of the seaweeds having an inhibitory effect on its growth. In general, the Gram positive bacteria appear to be more sensitive to treatment than the Gram negative bacteria. This is in agreement with Reichelt and Borowitzka (1984), Allen and Dawson (1960) and Rao and Parekh (1981).

In a study to determine whether the antimicrobially active seaweeds were in fact devoid of the test microorganisms in nature, an electron microscopy study was carried out. This study revealed the presence of both rod and coccal shaped organisms on the surfaces of the three Phaeophyceae studied. As the spring figures show varying degrees of activity against *S.aureus* and the *Micrococcus* sp. with no activity against *B.subtilis*, fewer coccal-shaped organisms, and more rod-shaped organisms would be expected on the micrographs, this however was not the case. The concentration of organisms appear to be similar on all parts of the seaweed, this may suggest that any antibiotic compounds produced occur evenly in the base, middle and tip of the thalli of *Z.subarticulata*, *E.biruncinata* and *S.heterophyllum*.

Some seaweeds were assessed for their antineoplastic properties using the cancerous cell line, mouse melanoma-350, and the normal cell line, LLCMK. The three Phaeophyceae tested, *Z.subarticulata*, *E.biruncinata* and *S.heterophyllum* all reduced the growth of both the mouse melanoma-350 and the LLCMK cells, this is an undesirable effect, whereas the Chlorophyceae, *Ulva rigida*, *Caulerpa filiformis* and *Codium duthieae*, all stimulated the growth of the LLCMK cells and reduced the growth of the cancerous cells to varying degrees. In other parts of the world, the green algae seem to have gained most attention in this field of study. A number of these algae have been shown to exhibit significant cytotoxic activity, with some of the active compounds being identified. The brown algae have not been entirely neglected, *Stypopodium zonale* has been found to be non-toxic to mice and expanded the life-expectancy of mice injected with P388 lymphocytic leukemia or Ehrlich ascite tumour cells. One each of the eastern Cape red and brown seaweeds and *U.rigida* and *C.filiformis* (both greens) were then used in a cytotoxicity study using Brine shrimp. None of these were found to be toxic after 30 hours at a concentration of  $1000\mu\text{g ml}^{-1}$ .

The antiviral studies against Polio virus Type 1 Strain L-Sa also showed some promising activity. The Phaeophyceae, *Dictopteris macrocarpa* and *Dictyota naevosa* both reduced the virus titre, *D.naevosa* as much as 4.0 logs. The Chlorophyceae *Ulva rigida* and *Halimeda cuneata* were active against the virus, as was *Hypnea spicifera*. *Laurencia glomerata* only prevented a change in virus titre. Previous studies have shown that the active compounds of most seaweed extracts found to be active against mammalian

viruses have been polysaccharides. Inhibition was also found to be greater with pretreatment than posttreatment of cells. This would limit the usefulness of the compounds. Experiments with pre- and post-treatment however were not carried out on the eastern Cape seaweeds.

From this project, it appears that the seaweeds of the eastern Cape coastline have great biomedical potential. The coast of South Africa varies greatly, harbouring different species which have grown under vastly different conditions. This project shows that the potential exists for further studies on the biological activity of extracts of South African seaweeds.

## Appendix A

### Recipes used in chapters II and III.

#### Phosphate buffer (0,1M pH 7,5)

Make up as follows

2,8218 g of  $K_2HPO_4$

0,5171 g of  $KH_2PO_4$

Make up to 100 ml using distilled water. This gives a solution with pH of 7,4. Adjust the pH to 7,5 using NaOH.

#### Nutrient Broth – Diagnostica Mark 41993619

Dissolve 8 g in 1ℓ of distilled water. Mix well and sterilize at 121°C for 15 min in an autoclave.

#### Nutrient Agar – Difco 602074

Dissolve 23 g in 1ℓ of distilled water. Mix well and sterilize at 121°C for 15 min in an autoclave. Bacto Nutrient agar contains:

Bacto-Beef extract      3 g

Bacto-Peptone          5 g

Bacto-Agar              15 g

#### Wickerham's Agar

Malt extract            3 g

Yeast extract           3 g

Bacto-Peptone          5 g

Glucose                 10 g

Agar                      20 g

Wickerham's agar is prepared by dissolving the above in 1ℓ of distilled water and mixing well then sterilizing at 121°C for 15 mins in an autoclave.

#### TTC Plates (2,3,5-triphenyltetrazolium chloride)

A layer of nutrient agar is poured, allowed to set, then the TTC\bacteria\agar overlay is prepared in the following way:

250 ml nutrient agar

10 ml overnight bacterial culture

5 ml TTC solution at a concentration of 0,5 mg.ml<sup>-1</sup>

This is poured over the nutrient agar base and allowed to set afterwhich wells can be punched in the double layer of agar.

## Appendix B

### Recipes used in chapter IV

#### Phosphate buffer (0,1M pH 7,3)

Make up as follows:

Soln 1: 35,814 grams  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$

Soln 2: 13,610 grams  $\text{KH}_2\text{PO}_4$

To achieve a molarity of 0,1 and a pH of 7,3 these solutions are mixed in the ratio 80 parts Solution 1: 20 parts solution 2.

#### Buffered fixative (gluteraldehyde)

A 2.5% solution of buffered gluteraldehyde is prepared by making up 20 mls of 25% gluteraldehyde (ultrastructure grade) to 100 mls with buffer prepared as above. If any cloudiness is observed in the buffered gluteraldehyde some polymerization of the stock solution has probably taken place and it should be re-distilled or fresh supplies obtained.

#### Dehydrating Agents

##### Ethanol

The required concentrations of ethanol are prepared by diluting stock 95% ethanol with distilled water according to the following table:

|                  |                               |          |          |
|------------------|-------------------------------|----------|----------|
| 160 mls 95% EtOH | +340 mls $\text{H}_2\text{O}$ | =500 mls | 30% EtOH |
| 260 mls          | +240 mls                      | =500 mls | 50% EtOH |
| 370 mls          | +130 mls                      | =500 mls | 70% EtOH |
| 420 mls          | + 80 mls                      | =500 mls | 80% EtOH |
| 475 mls          | + 25 mls                      | =500 mls | 90% EtOH |

#### Chloroform-based adhesive for SEM specimens.

Strip off about 1m of standard 10 mm wide clear adhesive tape e.g. Cellotape, and crumple it up into a small enough ball to fit into a 30 mm wide opening of a 100 ml reagent bottle. Add 20 ml chloroform and shake. The adhesive layer will gradually dissolve in the chloroform leaving a piece of non-adhesive tape which can be removed from the bottle. The remaining liquid is the chloroform-based adhesive. It can be stored indefinitely in a tightly stoppered bottle. If by evaporation of the chloroform, it becomes too thick, it can simply be diluted with additional chloroform. Further adhesive can be prepared by adding as much more tape as may be required from time to time.

## Appendix C

### Recipes used in Chapter IV

#### Tissue Culture Medium

Eagle's MEM which contains Hank's salt and glutamine was used. One packet of EMEM makes 10  $\ell$ s.

Per  $\ell$  of Milli-Q water add the following to the medium:

|         |                    |
|---------|--------------------|
| 0.05 g  | Ascorbic Acid      |
| 0.01 g  | Serine             |
| 0.006 g | Glycine            |
| 0.75 g  | NaHCO <sub>3</sub> |
|         | Antibiotics        |

#### Antibiotics:

Per  $\ell$ , add 0.5 X 10<sup>5</sup> IU Penicillin

0.5 X 10<sup>5</sup>  $\mu$ g Streptomycin

[One vial each of Penicillin and Streptomycin is diluted with 100 ml Milli-Q water, 5 ml of mixture per  $\ell$  of tissue culture medium is used].

#### Trypsin:

Per  $\ell$  of Milli-Q water add:

|                     |                      |
|---------------------|----------------------|
| 8,0 g               | NaCl                 |
| 0,4 g               | KCl                  |
| 1,0 g               | D-glucose            |
| 0,58 g              | NaHCO <sub>3</sub>   |
| 0,2 g               | EDTA                 |
| 46,7 g              | Trypsin              |
| 0,02 g              | Phenol Red           |
| 1 X 10 <sup>5</sup> | IU Penicillin        |
| 1 X 10 <sup>5</sup> | $\mu$ g Streptomycin |

#### Procedure for Freezing Cells:

##### Tissue Culture Freezing Medium:

To the medium add: 20% FCS

10% DMSO

Antibiotics as before

Sterile filter the freezing medium and store frozen until required.

##### Freezing Cells:

Use 75% confluent cells. Trypsinize the cells, then incubate until the cells lift off the flask surface.

Transfer the cells to sterile centrifuge tubes, and centrifuge at 1000g for 10 min. Resuspend the cells in 2 ml freezing medium which has been filtered. Dispense into vials, and freeze in liquid N<sub>2</sub> vapour for 1 hr.

Freeze in liquid N<sub>2</sub> until required.

## Appendix D

### Recipes used in chapter VII.

Fixative: 0.01 M PBS, pH 7,3, with Formalin.

Make up as follows:

2,18g  $\text{Na}_2\text{HPO}_4$

0,728g  $\text{NaH}_2\text{PO}_4$

Make up to 2ℓ using distilled water

Add: 17,2g NaCl (0,86%)

100ml Formalin (5%)

Stain: Crystal violet in Ethanol

0,1% Crystal violet in 50% Ethanol

### Titre Determination

| Dilution         | Observed | Accumulated |      | % Accumulated<br>(p+n)100 |
|------------------|----------|-------------|------|---------------------------|
|                  | + -      | p(+)        | p(-) |                           |
| 10 <sup>-4</sup> | etc      |             |      |                           |
| 10 <sup>-5</sup> | etc      |             |      |                           |
| 10 <sup>-6</sup> | etc      |             |      |                           |

$$\text{TCID}_{50} = g + \Delta \frac{(\% + \text{'ve at dilution above 50\%}) - 50}{(\% + \text{'ve at dilution above 50\%}) - (\% + \text{'ve at dilution below 50\%})}$$

$$\text{NI} = \text{TCID}_{50} (\text{control}) - \text{TCID}_{50} (\text{exp.})$$

Key:  $\text{TCID}_{50}$  – Tissue Culture Infective Dose 50% End Point  
g – -'ve Dilution above 50% End Point  
 $\Delta$  – log dilution factor  
NI – Neutrillization Index

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